Intraspecific Genetic Variation in New Zealand's Endemic Frog, *Leiopelma hochstetteri*

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Abstract

New Zealand's endemic frog species, *Leiopelma hochstetteri*, is a good model for studying the effects of population fragmentation on patterns of genetic diversity. *L. hochstetteri* inhabits streambeds that are becoming increasingly isolated due to urban and agricultural development. The potential for autosomal divergence between populations of *L. hochstetteri* is already apparent from the great extent of cytogenetic diversity within the species. Different populations exhibit variable numbers of B-chromosomes, ranging from none in some populations to as many as fifteen. Past studies on B-chromosomes in other taxa have indicated these karyological elements can affect gene flow between populations, resulting in genetic isolation and differentiation.

This study investigated sequence variation in 600 bps of the mtDNA gene cytochrome b to determine the phylogenetic relationships ~~between~~ among 17 populations of *L. hochstetteri*. The phylogeny and population structure was investigated using different methods: phylogenetic reconstruction, a minimum spanning network, and analysis of molecular variance. The sequence variation between *L. hochstetteri* and the outgroup, *L. archeyi*, was exceptionally high (20%) for a sister species. *L. hochstetteri* was found to be highly structured at the population level (64%, $\Phi = 0.740$, $p = 0.001$) suggesting little or no gene flow ~~between~~ among geographically close populations. Phylogenetic relationships above the population level were ambiguous.

The presence or absence of B-chromosomes appears to have no affect on population structure in *L. hochstetteri*. The molecular phylogeny indicates that B-chromosomes arose multiple times in *L. hochstetteri* and ~~that B-chromosomes~~ evolved ~~in this species~~ earlier than previously thought. Finally, in consideration of this new evidence on the phylogeny and karyology of *L. hochstetteri*, recommendations are made concerning the prioritisation of populations for conservation.

Chapter One

Introduction

This chapter presents background information on the ideas and issues discussed in this thesis. The chapter begins with a discussion of the problems surrounding the designation of species using genetic data. New Zealand's native frogs are introduced and the ecology and general biology of *Leiopelma hochstetteri* is discussed. The presence of B-chromosomes in *L. hochstetteri* and their evolutionary significance in general is introduced. Finally, there is a justification of the use of the mitochondrial gene, cytochrome b, as a molecular marker and the aims of the study.

1.1 Speciation

Genetic divergence through allopatry has long been accepted as the most compelling mechanism of speciation (Dobzhansky 1935; Mayr 1963). Habitat fragmentation leads to genetic divergence between populations (Joseph *et al.* 1995), and eventually results in reproductive isolation. Under the biological species concept (Dobzhansky 1935; Mayr 1963) any level of reproductive incompatibility between populations is evidence of a speciation event. In practice, the reproductive status of different populations is often unknown and, with the popularity and increasing ease of molecular studies, the amount of genetic difference between populations is commonly used as an analogous index of divergence.

The phylogenetic species concept (Eldredge and Cracraft 1980; Cracraft 1983) uses genetic data and phylogeny to describe species units. The species criteria is based on ~~genetic~~ monophyly instead of the reproductive compatibility between populations. Although the phylogenetic species concept has great utility, there are some caveats to consider. Smaller populations have a greater chance of being designated species units because they are likely to be monophyletic (Avise and Wollenburg 1998). Beneficial and neutral mutations accumulate quickly in small populations, resulting in an increased number of synapomorphies compared to larger populations. Large populations tend to be paraphyletic with their close relatives (Avise and Wollenberg 1998), and will not be designated as distinct species as readily. The presence of a polytomy, where multiple species radiate almost simultaneously (Maddison 1989), can also lead to confusion in the designation of phylogenetic species (Hoelzer and Melnick 1994). In a polytomy, the species that is most genetically derived may not have been the first one to become reproductively isolated (Hoelzer and Melnick 1994; Walsh *et al.* 1999). This incongruity between genetic divergence and reproductive isolation leads to an incorrect phylogeny. Another difficulty is determining the amount of genetic divergence required to designate a species. Using mtDNA data, genetic resolution can be so precise as to resolve family units and even individuals. Such precision makes it difficult to separate significant groupings from trivial ones (Avise and Wollenberg 1998).

The difficulties in applying the phylogenetic species concept can be resolved in two ways. Increasing the amount of genetic information to include multiple genes or types of genetic assay can separate significant phylogenetic groups from genetic noise (Avise and Wollenberg 1998). Perhaps species boundaries, as defined by the biological species concept, are not the divisions that should be considered. There are other possibly more useful and informative divisions between genetic groups.

Moritz (1994) has introduced the idea of "evolutionarily significant units" (ESU). Evolutionarily significant units are genetically differentiated populations within a species which are critical to the maintenance of its genetic diversity. In defining ESUs, emphasis is placed on past population structure rather than current adaptive variation to ensure conservation of critical populations (Moritz 1994). The idea of an ESU is applied to conservation in an effort to save the genetic identity of a species. However, ESUs, as genetically distinct populations, also represent potential species. Using ESUs, the value of all genetic groups is evaluated without having to define a species boundary.

1.2 New Zealand's native frogs

The family Leiopelmatidae is regarded as the ancestral extant family in the order Anura (Cannatella 1985; Green 1989). The family includes both the genus *Leiopelma*, which contains all New Zealand's native frogs, and the North American species *Ascaphus truei*. Although *Ascaphus* and *Leiopelma* both represent "primitive" lineages, isozyme studies have defined *Leiopelma* as the ancestral of the two by a margin which could place it into a separate family (Green 1989, 1993). Its distinction as the most basal genus of all Anura highlights the phylogenetic and evolutionary significance of *Leiopelma*.

The genus *Leiopelma* consists of three extinct and four extant species, including *L. hochstetteri*, *L. archeyi*, *L. hamiltoni*, and *L. pakeka* (Worthy 1987; Bell 1994, 1998). Before the arrival of the Maori in New Zealand, all seven species were present. The Polynesian rat was most likely responsible for the disappearance of the three extinct frog species as well as a significant range reduction in the four extant species (Worthy 1987). According to subfossil remains, *L. hochstetteri* once ranged over the entire North Island and as far south as Punakaiki on the South Island (figure 1.1A). Now, *L. hochstetteri* only lives in isolated patches across the North Island (Worthy 1987). *L. hamiltoni* and *L. pakeka* are currently found on small offshore islands in the Marlborough Sounds area, but were once as widely distributed as *L. hochstetteri* (Worthy 1987) (figure 1.1B). *L. archeyi* is restricted to the Coromandel region, but no subfossil evidence indicates it ever had a wider distribution (Worthy 1987). The population sizes and distributions of these frogs have been reduced to such an extent that all species are now endangered (Newman 1996).

The most threatened of New Zealand's frogs are the two species restricted to offshore islands. *Leiopelma pakeka* is a new species which was recognised only two years ago (Bell *et al.* 1998). *L. pakeka*, found on Maud Island in the Cook Strait, was originally thought to be

the same species as *L. hamiltoni*, ~~which lives~~ on Stephen's Island (Stephenson 1961). Isozyme differences between the two island populations ~~revealed~~ suggested they were distinct species (Bell *et al.* 1998). Because of this division *L. hamiltoni*, which has fewer than 150 individuals (latest census, unpublished), is one of the rarest frogs in the world (Bell 1985; Newman 1990; Brown 1994). Since the recognition of *L. pakeka*, there has been increased awareness of the need to preserve *L. hamiltoni* and *L. pakeka*, and a renewed interest in all of the *Leiopelma* species.

Considering the recent discovery of the cryptic species *L. pakeka*, a need exists for more extensive studies on intraspecific genetic variation in the genus *Leiopelma*. The identification of genetically unique populations is especially important given the endangered status of these frogs. Specifically, *Leiopelma hochstetteri*, the most widespread and potentially diverse native frog, needs attention.

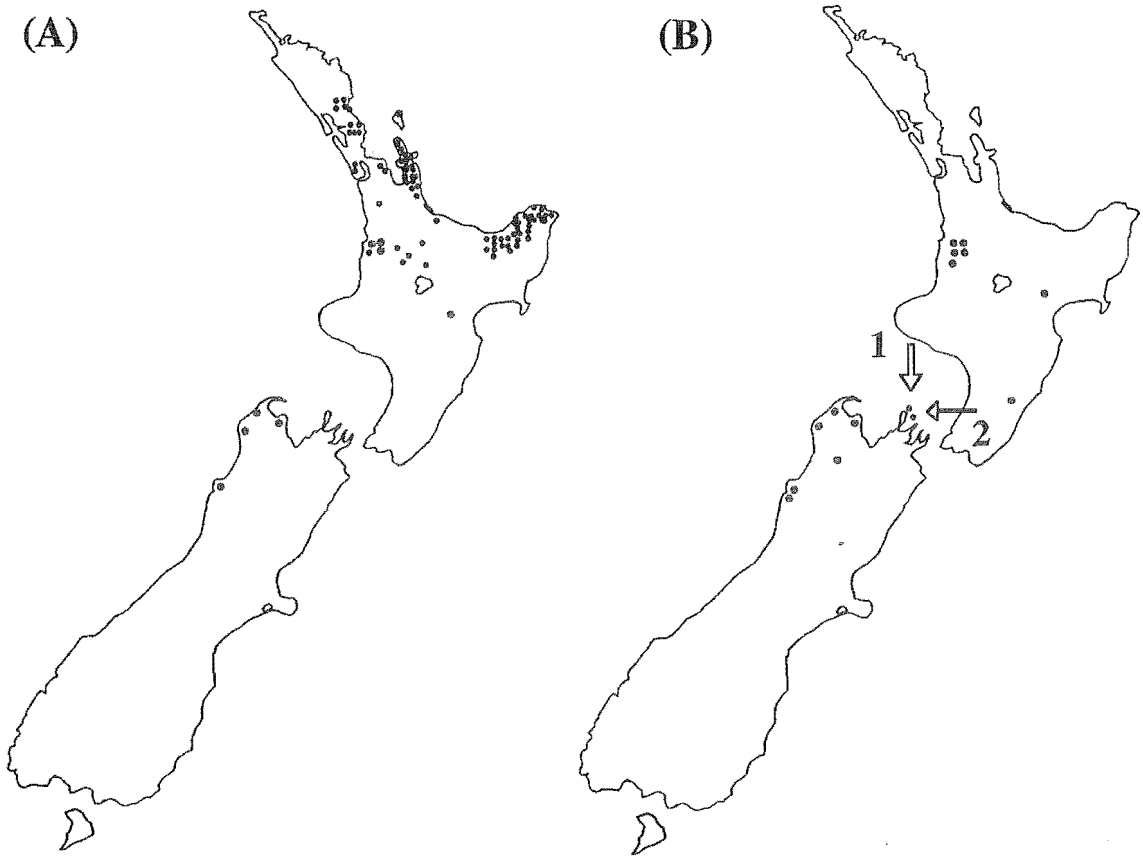


Figure 1.1 Past and Present Distributions of *Leiopelma* (A) *L. hochstetteri* (B) *L. hamiltoni* (1) and *L. pakeka* (2). Black dots denote present populations, grey dots extinct populations. Data from Worthy (1987) and Newman (1996).

1.3 Hochstetter's frog

Leiopelma hochstetteri, or Hochstetter's frog, was the first of the native frog species to be formally recognised (Fitzinger 1861). Although the most abundant of New Zealand's endemic frogs, *L. hochstetteri* is categorised as "at risk" by the Red Data Book categories of the IUCN (International Union for the Conservation of Nature), and has been protected since

1922 (Bell 1994, 1985). The species currently lives in fragmented populations across the North Island and on Great Barrier Island (figure 1.2) with the highest population density on the Coromandel Peninsula (Newman 1996). Subfossil remains of *L. hochstetteri* appear throughout the North Island and even on the northern half of the South Island, indicating its range was once much wider (figure 1.1A)(Worthy 1987). Recently, a new population of *L. hochstetteri* was discovered at Whareorino in the King County region (Thurley and Bell 1994). Such ignorance concerning even its distribution reiterates the need for more research on this frog.

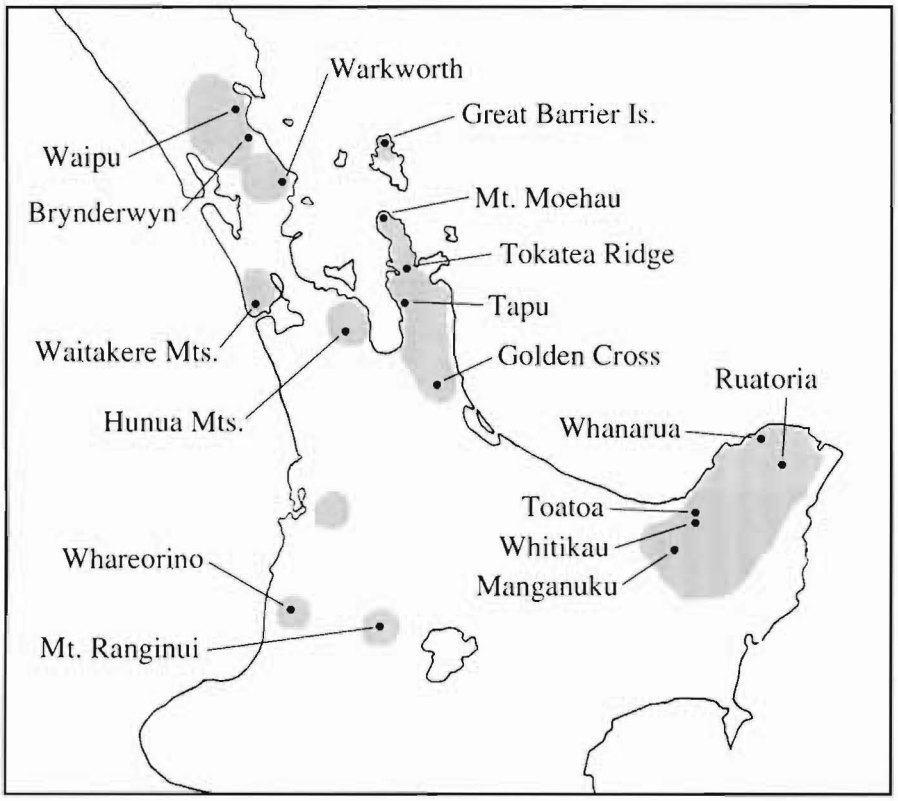


Figure 1.2 Current distribution of *L. hochstetteri*. DNA samples in this study were collected from the labelled locations.

The habitat preferences of *L. hochstetteri* differ from those of its terrestrial cousins. *L. hochstetteri* is a specialist preferring narrow zones along streambeds (Green and Tessier 1990)(figure 1.3). Although the frogs are sensitive to microhabitat disruptions like streambed silting (Green 1990), populations can thrive in streams surrounded by inhospitable environments such as commercial pine plantations (personal observation). Few studies exist documenting population densities and behaviour of these frogs. However, the frogs are known to occur in densities of up to 50 per 100m of stream (Green and Tessier 1990). Dispersal capabilities of *L. hochstetteri* are unknown but are thought to be limited since the frogs are at least partially dependent on water and individuals have been found repeatedly under the same rock (Tessier *et al.* 1991). Because *L. hochstetteri* is a habitat specialist with

limited dispersal, it would not be surprising to find genetic divergence between populations resulting from lack of gene flow.

As well as being morphologically and ecologically distinctive from other *Leiopelma* (Bell 1994), *L. hochstetteri* is also karyologically and biochemically divergent (Daugherty 1981; Green 1988, 1991; Green *et al.* 1987, 1993). *L. hochstetteri* is known for its extraordinary variation in B chromosome number, which have not been found in other members of the genus (Green 1988, 1988b, 1991; Green *et al.* 1987, 1993). Isozyme data indicate *L. hochstetteri* separated from the rest of the *Leiopelma* species during the Miocene (15 mya) which is long before the other *Leiopelma* species diverged in the Pliocene (Daugherty 1981). Two different isozyme studies support *L. hochstetteri* as highly divergent within the genus, with a Nei's unbiased genetic distance (Nei 1972) of 1.04 and 1.158 (Daugherty 1981; Green 1989, respectively) between *L. hochstetteri* and the other *Leiopelma*. This amount of genetic distance was enough for Green (1989) to propose putting *L. hochstetteri* into a separate genus.

Molecular studies have yet to determine whether a relationship exists between karyological diversity and genetic diversity in *L. hochstetteri*. Some of the difficulties in answering this question stem from the nature of B-chromosomes in general and specifically their manifestation in *L. hochstetteri*.

Figure 1.3 ***L. hochstetteri* habitat** the Little Manganuku River on the East Cape Peninsula. Photo by author

1.4 B-chromosome variation in *L. hochstetteri*

B-chromosomes, or supernumerary chromosomes, are rare in anurans (Jones and Rees 1982; Green 1991), but *L. hochstetteri* exhibits among the most extensive variation in B-

chromosome number of any vertebrate (Green 1988). The normal karyotype of *L. hochstetteri* is $2n$=22 (Stephenson *et al.* 1972), but as many as 15 additional B-chromosomes have been found in one individual (Green 1993). Morescalchi (1967) first identified B-chromosomes in *L. hochstetteri*, but they have been extensively studied since by Green and his colleagues (Green 1988, 1991; Green *et al.* 1987, 1993).

In addition to the normal B-set of supernumerary chromosomes, *L. hochstetteri* has a female-specific univalent sex chromosome. The sex chromosome is present in all populations of *L. hochstetteri* with the exception of those found on Great Barrier Island (Green 1988). To prevent confusion, the sex determining B-chromosome will be referred to as the univalent sex chromosome and the term "B-chromosome" will refer to the rest of the B-set.

Of the 12 populations surveyed to date, six exhibit varied number of B-chromosomes (Green 1988, 1991; Green *et al.* 1987, 1993). A summary of these finding is provided in table 1.1.

Table 1.1. Distribution of B-chromosomes *in L. hochstetteri* Data includes the number of individuals karyotyped, the mean number of chromosomes over all individuals and the range of chromosome number. Data does not include the univalent sex chromosome found in all populations except Great Barrier Island.

Location	N	Mean	Range	Source(s)[1]
Golden Cross	3	0	0	f
Great Barrier Is.	9	0	0	f
Hunua Mts.	7	1.3	0-3	e, f
Mt. Moehau	7	11.4	9-15	e, f
Mt. Ranginui	6	0	0	f
Tapu	13	7.1	1-12	d, e, f
Toatoa	12	0	0	e, f
Tokatea Ridge	14	3.7	1-10	b, e
Waipu	4	0	0	f
Waitakere Mts.	9	4.0	2-11	f
Warkworth	20	0.6	0-11	a, b, c, d, e
Whanarua	3	0	0	f

[1] Data compiled from all known sources: a) Morescalchi 1967; b) Stephenson *et al.* 1972; c) Green *et al.* 1984; d) Green *et al.* 1987; e) Green 1988; f) Green *et al.* 1993.

Remarkable variation in B-chromosome number exists both within and among populations. Not only are these B-chromosomes diverse in number, they are also morphologically diverse. The B-chromosomes vary in size and structure with eleven distinct types (Green *et al.* 1993). The number of B-chromosomes and their characteristics appear to have no correlation with the geographic relationships of the different populations (Green *et al.* 1993).

Because of their hypervariability, difficulties arise in determining the evolutionary history of these karyological elements. Although B-chromosomes can evolve through interspecific crosses (McVean 1995), southern hybridization shows these B-chromosomes arose from the frog's own genome (Sharbel *et al.* 1998). According to Green *et al.* (1993), the univalent sex chromosome system, 0W/00, evolved from the heteromorphic ZW/ZZ sex

determining system still found on Great Barrier Island and in *L. hamiltoni* (Green 1988), but not *L. archeyi* (Green 1988b). The 0W/00 sex determining system evolved from ZW/ZZ through the fixation of ZZ homozygosity. Trisomy of the Z chromosome, ZZW/ZZ, would be cytogenetically indistinguishable from 0W/00. Green *et al.* (1993) hypothesizes that both forms were present before Great Barrier Island split from the North Island 10 000 years ago (Flemming 1975). After the split, ZW/ZZ became fixed on Great Barrier Island through drift, and 0W/00 was fixed on the North Island. Selection may have favoured ZZW over ZW through elimination of sex linked deleterious mutations on the Z chromosome, which would have been hemizygous in the ZW form (Green *et al.* 1993). Once the 0W/00 system became fixed on the North Island, the W chromosome was barred from recombination. Accumulating genetic change through Muller's ratchet (see section 1.5), it became more heterochromatic and increasingly isolated from the rest of the genome (Green 1990). Sharbel *et al.* (1998) found sequence homology between the univalent sex chromosome and the rest of the B-set. This evidence, coupled with evidence of similar morphology (Green *et al.* 1993), supports the emergence of the other B-chromosomes from the univalent sex chromosome (Sharbel *et al.* 1998).

The diversity of B-chromosomes in *L. hochstetteri* formed from the univalent sex chromosome during either one or more karyological events. Based on sequence similarity between B-chromosomes, Sharbel *et al.* (1998) argues for a single origin. However, Green *et al.* (1993) found morphological differences between the B-chromosomes in the Waitakere population and those from other locations, which would imply at least two separate chromosome mutations. The hypervariability of these chromosomes could easily obscure any evolutionary trend. However, comparing chromosome information with another index of relationship, such as sequence data, might elucidate the evolutionary relationship between B-chromosomes from different populations.

1.5 B-chromosomes and species evolution

Although chromosome changes have often been implicated in the isolation of different populations during speciation (reviewed in King 1993), the role of B-chromosomes in particular is still widely debated. The difficulties in understanding B-chromosomes stem from their high variability and indeterminate characteristics. Green (1991) said of B-chromosomes, "their major characteristic is their lack of general characteristics." Thus, it is common within the literature to find widely differing opinions concerning their impact on population genetic structure.

Generally, B-chromosomes are considered expendable parts of the genome that are not necessary for normal development (White 1973; Robinson and Roux 1985; Green 1991). B-chromosomes often originate from the A-set (Green 1991), but lose their coding regions to heterochromatinization through Muller's ratchet (Green 1990). Muller's ratchet refers to the process by which deleterious mutations accumulate in DNA that is barred from recombination

(Muller 1964). Although B-chromosomes are generally non-coding, Green (1987) has found evidence of transcription activity, bringing into question even the expendable nature of these karyological elements.

Many types of chromosome change, including B-chromosome formation, do not visibly contribute to changes in development or morphology (John 1981). However, B-chromosomes can have many invisible effects on a genome. Studies show the presence of B-chromosomes can be associated with reduced recombination, meiotic non-disjunction, depressed fertility, and restricted gene flow between populations (John 1973, 1981; Green 1991; McVean 1998). How often and to what extent these processes occur differ from study to study, further supporting the ambiguous nature of B-chromosomes.

If the presence of B-chromosomes leads to a loss of fitness through developmental disturbances or reduced fertility, it would be reasonable to assume that selection would eliminate them from the genome (John 1981). This does not occur because B-chromosomes are selfish genetic elements. Propagating themselves through segregation distortion in gametes (White 1973; Green 1991; McVean 1998), they maintain or even increase their numbers despite a decrease in population fitness. Because some species have a greater tendency to evolve B-chromosomes (King 1981), even if selection or drift eliminates them from a population they could reappear.

Loss of gene flow between populations can lead to fixation of different chromosome races between populations, and eventually speciation. The process begins with the emergence of different chromosome races within a genetically homogenous population (King 1993). Over many generations, some of the populations will accumulate fixed genetic differences, while others remain genetically similar. Genetic differences will continue to become fixed between populations until what were once populations of one species have become separate species (King 1993). Populations that diverged early from the parent species will have more synapomorphies than those that diverged later. This model applies to any type of chromosomal change, some of which have stronger isolating affects than others. Since the B-chromosomes' ability to restrict gene flow varies from case to case, the time-scale for the above model in *L. hochstetteri* is impossible to predict. However, comparing genetic relationships between populations with known karyological data will help determine what influence B-chromosomes have on gene flow in *L. hochstetteri*, and what class of speciation, if any, best represents the available data.

1.6 Past studies on the genetics of *L. hochstetteri*

Prior studies on genetic variation in *L. hochstetteri* have not been extensive, and have been restricted to isozymes and immunological evidence (Daugherty 1981, 1982; Green 1989, 1994) . The first study by Daugherty (1981) was a general analysis of isozymes within the genus and included only 16 *L. hochstetteri* from five populations. The study was admittedly not designed to investigate intraspecific variation, but found that differences between

populations of *L. hochstetteri* were roughly equivalent to that found in other vertebrates (Daugherty 1981). These findings were corroborated by a second study employing albumin variation to examine the same individuals (Daugherty 1982).

Using isozymes, Green (1989, 1994) has conducted more extensive studies specifically investigating intraspecific variation in *L. hochstetteri*. The first study examined 43 allozyme loci from 50 individuals representing six populations. Green (1989) found a clear pattern of relatedness between populations which suggested the current distribution of *L. hochstetteri* was the result of a radiation beginning on the Coromandel Peninsula and extending to the East Cape region. Green (1994) later increased the sample size to 96 specimens from 11 populations. Contrary to the results of the first study, the biochemical distinction between populations decreased. This is not surprising considering that as the number of individuals increases so does the chance of sampling rare genotypes shared between populations. Even though *L. hochstetteri* was generally genetically homogenous, three populations still stood out from the tight cluster formed by the rest. These populations were from Mt. Ranginui, Golden Cross, and the Hunua Mountains (Green 1994). Isozymes are a conservative estimate of biochemical variation, and the historical relationships between populations could be more efficiently investigated using a more variable marker, such as mitochondrial DNA sequence data.

Recently, a study by Holyoake *et al.* (1999, in press) on sequence variation in *Leiopelma* found evidence for divergence between populations of *L. hochstetteri*. *L. hochstetteri* showed a 0.66% sequence difference in 300 bps of the cytochrome b gene within samples from two populations on the Coromandel. The same study found that *L. pakeka* and *L. hamiltoni*, only recently considered different species, were genetically identical or nearly identical. This study suggests that mtDNA sequence analysis would be a good marker for the further investigation of evolutionary relationships among populations of *L. hochstetteri*.

1.7 The phylogenetic utility of mtDNA and cytochrome b

Mitochondrial DNA

The utility of mitochondrial DNA sequence analysis in determining relationships within and between species has long been recognised (Wilson *et al.* 1985). Mitochondrial DNA is predominately maternally inherited without recombination, so mutations accumulate at a higher rate than nuclear genes which undergo recombination (Wilson *et al.* 1985). Another advantage to maternal inheritance is the ability to follow specific mitochondrial lineages, and through these lineages the evolutionary history of population subdivisions and bottlenecks (Wilson *et al.* 1985).

Recently, the strictly maternal inheritance of vertebrate mtDNA has come into question. Two studies have found evidence of recombination between mitochondrial lineages in humans (Hagelberg *et al.* 1999; Walker *et al.* 1999). The results of one have been rescinded (Hagelberg 2000), and the validity of the other has come under attack. Walker *et al.*

(1999) found that the level of homoplasy between lineages greatly exceeds the amount expected considering mutation constraints and base compositional bias, and concluded recombination was the source of the discrepancies. Hay (2000) and Wallis (1999) recently have argued that the lack of knowledge about mutation, not recombination, is the answer to the problems with homoplasy in human mtDNA. Although more evidence needs to be gathered to confirm non-maternal inheritance, its possibility should still be considered. If recombination has occurred in *L. hochstetteri*, the effects would be an increased level of homoplasy and a reduction in genetic distance between lineages. The consequences of reduced genetic distance between lineages would be an underestimation of population structure, and this would make the phylogeny more conservative.

Cytochrome b

The mitochondrial cytochrome b gene was chosen for phylogenetic analysis. Cytochrome b has great utility as a genetic marker for intraspecific relationships due to its high mutation rate (Meyer 1994). However, a high mutation rate in a coding gene has drawbacks including early saturation of the third codon positions and little variation in first and second codons (Meyer 1994). Saturation is a problem when distantly related taxa are compared because it will obscure the phylogenetic signal through overprinting (Meyer 1994). Since this study is concerned with intraspecific genetic variation only, and not distantly related taxa, the limitations of saturation will have little impact. More importantly, the presence of saturation and base compositional bias can be tested and taken into account using most phylogenetic analysis packages.

Cytochrome b was a desirable marker not only because of a high mutation rate, but also because the study by Holyoake *et al.* (1999, in press) had found genetic variation within *L. hochstetteri* using that marker. Furthermore, Using the same molecular marker as the Holyoake *et al.* study makes it possible to compare genetic variation within *L. hochstetteri* to the intraspecific variation found in other *Leiopelma*.

1.8 Objectives of this study

In this study, I investigate the phylogenetic relationships ~~between~~ among different populations of *L. hochstetteri* using sequence information from the mtDNA cytochrome b gene. I attempt to reconcile karyological data with population genetic structure as well as elucidating historical relationships between populations. I also consider the implications of both chromosomal and autosomal genetic divergence in an effort to identify populations that require special consideration for conservation due to their unique genetic composition.

Chapter Two

Materials & Methods

This chapter describes the materials and methods used in the study. The chapter begins with a description of where and how the samples were collected. Details of primer design, PCR, and the general preparation of the samples for RFLP analysis and sequencing follows. Finally, a description is given of the different programs and types of statistics used to interpret the data.

2.1 Reagents

A list of all reagents and solutions used for this study is found in appendix A. Only double distilled water (ddH_2O) was used to make solutions, and all solutions were sterilized by autoclaving. All glassware and disposables such as tips and microfuge tubes were also autoclaved before use. Pipettes used for PCR reactions were sequestered for that purpose only to prevent cross contamination.

2.2 Sample Collection

Dr. David M. Green (Repath Museum, McGill University, Canada) provided 68 samples for this study. These samples consisted predominately of liver and muscle tissue and were stored at -80°C. Appendix B lists the sample number, location, and karyotype, if known, for each sample.

Additional samples were collected in February of 2000 on the East Cape Peninsula. Toe clips were taken on site limiting the time the frogs spent out of the stream. Using a sterile scalpel, the second toe on the right hind foot was removed and stored in 100% ethanol. The scalpel was sterilized with 100% ethanol between frogs, and blades were replaced frequently to ensure a sharp edge. All work was carried out under the approval of the Animal Ethics Committee (application 2000: 14 - Intraspecific genetic variation in New Zealand's endemic frog *L. hochstetteri*) and with the permission and cooperation of the Department of Conservation East Cape and Bay of Plenty Conservancies. A description of each collection site and the sample numbers of the frogs can be found in appendix B.

2.3 Sample Preparation

A. DNA Extraction

DNA was extracted using a basic Chelex extraction (Walsh 1991). Approximately 2 mm^2 of tissue was suspended in 300µl of digestion buffer containing 5% Chelex (see appendix

A for all solutions). Proteinase K and RNase were added to a final concentration of 100µg/ml and the samples were incubated overnight at 37°C. The samples were centrifuged at 13,000rpm to precipitate debris. The supernatant was transferred to a fresh tube and an equal volume of 5% Chelex in TE was added. The sample was centrifuged once more at 13,000rmp, and the supernatant removed and stored at -80°C. Prior to amplification, the DNA was diluted 1:10 in ddH_2O as a working stock.

B. Primer Design

Primers were designed for the study because the commonly used Kocher *et al.* (1989) primers did not work on all *L. hochstetteri* individuals. Cytochrome b sequences from four species [*Xenopus leavis* (Roe 1985), *Salmo salar* (NC 001960), *Gadus morhua* (NC002081), *Alligator mississippiensis* (NC 001922)] were downloaded from GeneBank, or copied from a publication, and aligned using ClustalW 1.7 (Thompson *et al.* 1994). The primers were designed by identifying regions of the cytochrome b gene that were conserved between all four species (figure 2.1).

```
                16943                    16372      17258                    17284
X. leavis       ATGAAACTTCGGCTCTCTTCTAGG.....TGAATTGGAGGTCAACCAGTAGAAGA
A. miss.        ATGAAACTTTGGATCACTACTAGG.....TGAATCGGAGGCCAACCAGTAAACCC
S. salar        ATGAAACTTTGGCTCACTCTTAGG.....TGAATTGGAGGCATACCCGTGGAACA
G. morhua       ATGAAATTTTGGCTCTCTTCTAGG.....TGAATTGGAGGCGTACCTGTAGAACA
                ****** ** ** ** ** ****     ***** *****    *** **  *
```

Figure 2.1 Primer design based on conserved regions of cytochrome b. The regions displayed are for the forward primer and reverse primer respectively. Astrices denote base pairs shared between all individuals. The base pair numbers are taken from the *X. leavis* sequence (Roe 1985)

The region flanked by the primers corresponds to base pair numbers 16373-17257 of the *Xenopus laevis* mtDNA sequence (Roe 1985). Primers designed for this study (table 2.1) amplified a 942bp segment of the cytochrome b gene.

Table 2.1. Primer Sequences

Primer Name	*Sequence (5'-3')*
JHB1F	ATGAAACTTCGGCTCTCTTMTRGG
JHB36R	TCTTCTACTGGTTGACCTCCAATTCA
JHB1FT7	GTAATACGACTCACTATAGGGCATGAAACTTCGGCTCTCTTMRGG
JHB36RT3	AATTAACCCTCACTAAAGGGTCTTCTACTGGTTGACCTCCAATTCA
T7-IRD800	GTAATACGACTCACTATAGGGC
T3-IRD800	AATTAACCCTCACTAAAGGG

C. PCR Amplification

To amplify samples for sequencing, primers with bacterial promoter regions were used. A T7 promoter region was added to the 5' end of the forward primer (JHB1FT7) and a T3 promoter region was added to the 5' end of the reverse primer (JHB36RT3). These promoter regions were included so that a generic T7 and T3 flourescently labeled primer (T7-IRD800 and T3-IRD800 from MWG Corporations) could be used for automated sequencing on the LI-COR DNA sequencer model 4000L (LI-COR Corporation, see section 2.5.A).

PCR amplifications (Saiki *et al.* 1988) were carried out in 25μl reactions containing 0.4μM of each primer, 0.1mM dNTPs, 1XPCR buffer (100mM Tris-HCl, 15mM $MgCL_2$, 500mM KCl, pH8.3, Beohringer Mannheim), 1 unit *Taq* enzyme, and approximately 50ng of template DNA. Thermocycling was carried out as follows:

1. 94.0°C 1 min 30 sec
2. 94.0°C 0 min 30 sec
3. 60.0°C 0 min 30 sec
4. 72.0°C 0 min 45 sec
5. Go to 2 and repeat for 32 cycles
6. 72.0°C 4 min 0 sec

When the program finished, the samples were stored at 4°C. Following amplification, 5µl of PCR product was quantified using agarose gel electrophoresis alongside a molecular marker (p-Bluescript KS cut with *Dra* I and *Pvu* II). The DNA fragments were visualized using ethidium bromide stain under UV light (260-320nM). For each set of amplifications, a negative control was made using water instead of DNA. If the negative control showed signs of amplification product, all PCR reactions in that set of amplifications were discarded.

DNA Precipitation

The DNA from the PCR reactions was purified by isopropanol precipitation (Sambrook, Fritsch and Maniatis 1989). One volume of 4M ammonium acetate and two volumes of isopropanol were added to the reactions and mixed well by inversion. The samples were incubated at -20°C for at least one hour, and were centrifuged at 14,000rmp for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed with ice cold 70% ethanol. The pellet was air-dried and resuspended in 15μl of TE pH8.0.

2.4 RFLP Pilot Study

To determine whether there was enough genetic variation in the cytochrome b gene to resolve phylogenetic relationships between populations of *L. hochstetteri*, a pilot study employing PCR-RFLP analysis (Hillis, Moritz and Mabel 1996) was conducted. In this study, the 942bp region was amplified using primers JHB1F and JHB36R (see table 2.1) as described in section 2.3.C. The PCR product was digested with two restriction enzymes, *Sau* 3A and

Hae III, to determine whether there was restriction site variation, and therefore sequence variation, between populations.

A. Restriction Enzyme Digestion

Each restriction digest contained 8µl of PCR product with either 0.5 units of *Sau* 3A enzyme (Boerhinger Manheim) or 1.0 unit of *Hae* III (New England Biolabs), with 1µl of the manufacturer's recommended buffer for a total volume of 10µl. The DNA was digested over night at 37°C. Restriction products were visualized by running 5µl of digested product on a 2% agarose gel stained with ethidium bromide and visualized under UV light (260-320nM).

B. Creating Restriction Site Maps for *Sau* 3A and *Hae* III

The lengths of the restriction fragments were calculated by comparing the migration of each fragment to that of a restriction fragment of known length. The molecular marker used was p-Bluescript KS cut with *Hae* III. The physical distance each fragment migrated from the well was measured in millimeters on an enlarged photograph. The migration distance and restriction fragment size of the molecular marker, and the migration distance of the fragments with unknown size were imputed into SeqAid II 3.8 (Rufa and Rhoads 1989), which estimated the sizes of the unknown fragments. Once the approximate size of each band was known, a restriction size map for each enzyme was constructed.

C. Analysis of RFLP data

Every individual was scored for the presence or absence of each restriction site providing a matrix of binary data for phylogenetic analysis. The information from each enzyme was combined and the genetic difference between locations was calculated using a distance matrix in PAUP* 4.0 (Swofford 1998).

2.5 Sequencing

Samples were amplified for sequencing using primers JHB1FT7 and JHB36RT3 (see table 2.1) which contained the T7 and T3 promotor sequences. The addition of these promotor sequences allowed reamplification for direct sequencing with the LI-COR IRD 800 primers LI-COR Corporation). The primers and PCR reactions were kept in the dark because the IRD 800 primers are light sensitive. The samples were amplified and precipitated as described in section 2.3.C.

A. Sequencing Reactions

Sanger di-dioxy cycle sequencing was conducted using a Thermo Sequenase Cycle Sequencing Kit (USB Corporation). Reactions were performed using the manufacturer's reagents supplied in the kit (eg. ddNTPs, buffer, and Thermo Sequenase enzyme). The primer used was either a flouresently labeled LICOR IRD 800 T7 or T3 primer (see Table 2.1).

Each sequencing reaction included 200fmol amplified DNA, 2µl of ddNTP (either A, C, G or T), 0.25µl of buffer, 0.25µl enzyme, 0.25µl of primer and ddH_2O to a final volume of 4µl. The reactions were covered in one drop of mineral oil to prevent evaporation, and amplified using the following program.

1. 95.0°C 5 min 0 sec
2. 95.0°C 0 min 30 sec
3. 50.0°C 0 min 15 sec
4. 70.0°C 1 min 0 sec
5. Goto 2 and repeat for 29 cycles
6. 95.0°C 0 min 30 sec
7. 72.0°C 1 min 0 sec
8. Goto 6 and repeat for 9 cycles
9. Hold at 4.0°C

Reactions were stopped with the addition of 2µl Stop Solution (95% formamide, provided in kit) which preserved the single stranded state of the samples. Samples were stored at -20°C until use, which was never more than 24 hours after amplification to limit the amount of degredation. Prior to electrophoresis, samples were denatured at 95°C for 4 minutes, and immediately stored on ice.

B. Sequencing Gel Preparation and Electrophoresis

Sequencing bands were resolved in a 4% acrylamide gel. The gel contained 33.6g urea, 8ml 10XTBE, 6.4ml Longer Ranger Gel 50% stock solution (FMC Bioproducts) and ddH_20 added to a final volume of 80ml. The solution was gently heated to dissolve the urea and degassed for 5 minutes. Immediately prior to pouring 500µl of 10% ammonium persulfate and 40µl of TEMED were added to start polymerization.

The gel was cast between two 60cm long glass plates separated by 0.25mm spacers. The gel was allowed to set for 1.5 hours, or until it began to shrink away from the edges of the glass. Once polymerized, the gel was mounted and run to LI-COR specifications (Operator's manual). The running buffer was 1X TBE. The samples, 1.5µl of each, were loaded into a 48 well shark's tooth comb. The running parameters were 2,250 Volts, 68.8 Watts, and 30.6 mAmps. The gel was run for approximately 20 hrs. Samples were automatically sequenced using the LICOR software (Base ImagIR) and rechecked by eye.

C. Sequence Analysis

Sequences were aligned using ClustalW 1.7 (Thompson *et al.* 1994). The presence of each substitution was then double-checked against the original gel image. The ClustalW 1.7 alignments were downloaded into MacClade 3.08a (Maddison and Maddison 1999).

MacClade 3.08a was used to crop sequences and analyze base pair composition. PAUP* 4.0 (Swofford 1998) was used for phylogenetic analysis and tree building. Further details of the phyologenetic analysis are discussed in the appropriate sections of Chapters three and four.

Chapter Three

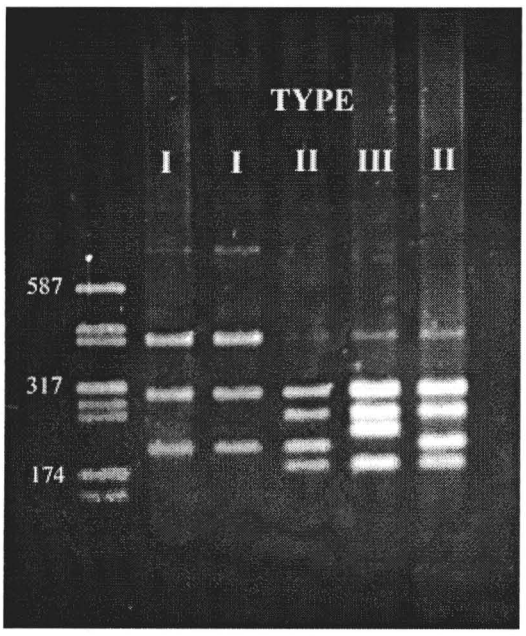
Inferring Phylogenies

In this chapter, the results of the RFLP pilot study are presented. The sequence data are then presented and a discussion of the phylogeny of *L. hochstetteri* commences with a review of the different assumptions implicit in an analysis of sequence variation. A review of the different criteria for inferring tree topologies from sequence data and confidence levels appears next.

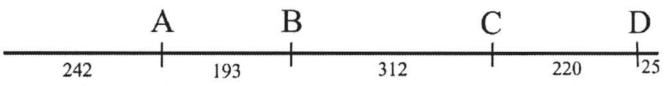
3.1 Suitability of cytochrome b

A pilot study investigating RFLP variation was conducted to test whether enough variation existed in the cytochrome b gene to resolve phylogenetic relationships within *L. hochstetteri*. Thirty-six individuals were sampled from fifteen populations. Four restriction sites exist for the *Sau* 3A enzyme (figure 3.1), only two of which were variable. Three restriction sites for *Hae* III existed, all of which were variable (figure 3.2).

(A)



(B)



(C)

TYPE	SITE A	B	C	D
I	−	+	+	+
II	+	+	+	+
III	+	+	+	−
IV	−	+	+	−

Figure 3.1. *Sau* 3A Digest of 942bp of the cytochrome b **(A)** agarose gel showing three of the four RFLP types produced by *Sau* 3A digestion. Marker is p-Bluescript KS cut with *Hae* III **(B)** Restriction map inferred from RFLP fragment sizes of the 942bp region **(C)** The presence/absence of each restriction site for each of the RFLP types

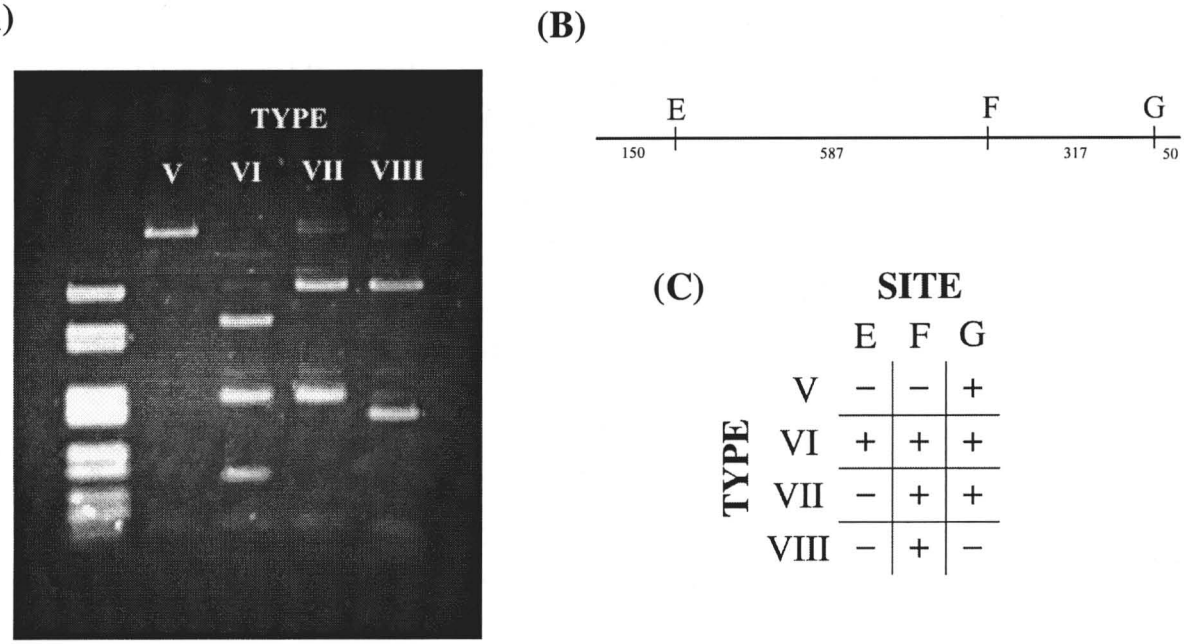


Figure 3.2. *Hae* III Digest of 942bp of the cytochrome b (A) agarose gel showing the four RFLP types produced by *Hae* III digestion. Marker is p-Bluescript KS cut with *Hae* III **(B)** Restriction map inferred from RFLP fragment sizes of the 942bp region **(C)** The presence/absence of each restriction site for each of the RFLP types

The pairwise genetic differences between populations, calculated using the combined data from both *Sau* 3A and *Hae* III, showed considerable genetic variation between populations (table 3.1). The mean character differences, calculated in PAUP* 4.0 (Swofford 1998), were transformed into percent nucleotide differences according to the method of Nei and Li (1979).

Table 3.1 Pairwise differences between RFLP haplotypes Above diagonal: percent nucleotide difference. Below diagonal: total character difference

Site	*Haplotype*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	Northland[1]	-	0.85	1.70	0.85	1.70	0.42	1.30	0.42
2	Golden Cross	2	-	0.85	0.85	1.70	1.30	1.30	0.42
3	Great Barrier Is.	4	2	-	0.85	0.85	1.30	0.20	1.30
4	Common[2]	2	2	2	-	0.85	1.30	0.42	0.42
5	N. Coromandel[3]	4	4	2	2	-	1.30	0.42	1.30
6	Waipu3036	1	3	3	3	3	-	0.85	0.85
7	Waitakere Mts.	3	3	1	1	1	2	-	0.85
8	Whareorino	1	1	3	1	3	2	2	-

[1] Brynderwyn, Waipu and Warkworth
[2] Hunua Mts., Mt. Ranginui, Whanarua, Whitikau, Tapu and Toatoa
[3] Mt. Moehau and Tokatea Ridge

There were eight distinct *Hae* III/*Sau* 3A RFLP haplotypes across the 15 sites sampled. The most common haplotype was found in 6 populations, Hunua Mts., Mt. Ranginui, Whanarua, Whitikau, Tapu and Toatoa. Mt. Moehau and Tokatea Ridge, both sites from the northern Coromandel Peninsula, shared a haplotype, as did all the sites from the Northland Conservancy. Waipu was the only population with two RFLP haplotypes, containing a unique haplotype as well as the Northland haplotype (refer to figure 1.2 for a site map).

RFLP analysis showed that there was enough genetic variation in the 942bp segment of the cytochrome b gene to warrant its use as a genetic marker to differentiate between different populations of *L. hochstetteri*.

3.2 Sequence analysis

Sequence data were obtained from 67 individuals. When aligned and double-checked for accuracy, at least 600 bps were obtained from every individual. The nucleotide sequences for each of the 27 mitochondrial haplotypes appears in appendix C, as well as the number of individuals represented by each haplotype.

A. Sequence homology

The presence of a nuclear copy of a mitochondrial gene can confound phylogenetic analysis by providing non-homologous sequences. Fortunately, there are warning signs that indicate the presence of a nuclear copy (Lopez *et al.* 1997). Multiple bands in a PCR reaction can be an indication of a nuclear copy of a gene, although multiple bands can also represent general non-specific amplification. A nuclear copy would also affect the sequencing reactions, causing multiple banding for different nucleotides and general poor quality. Another indication of a nuclear copy is sequence data that is highly divergent between supposedly closely related individuals (Lopez *et al.* 1997).

No evidence exists to indicate a nuclear copy of cytochrome b was sequenced in this study. When PCR amplifications were run on an agarose gel, there was no evidence of multiple banding (figure 3.3). All the sequences were genetically similar making it unlikely more than one copy was sampled. Furthermore, the high transition/transversion ratio obtained for the data (see section 3.2.D) is indicative of mtDNA sequence (Lopez *et al.* 1997)

B. Sequence alignment

Errors and ambiguities in sequence alignment result in incorrect phylogenies. When *L. hochstetteri* samples were sequenced with the forward primer, a two base pair deletion at nucleotides 156 and 157 was observed when these sequences were aligned with *L. archeyi*. However, this deletion was not a true deletion but the result of a compression in the sequencing gel. When the same samples were sequenced with the reverse primer, the two nucleotides were resolved. Unfortunately, the reverse sequences were not

collected for all individuals, so the two compressed base pairs are denoted as ambiguities in appendix C.

Besides the two compressed base pairs, no other indications of insertions or deletions were present in the data. It is unlikely that the sequences were mis-aligned since little intraspecific genetic variation exists.

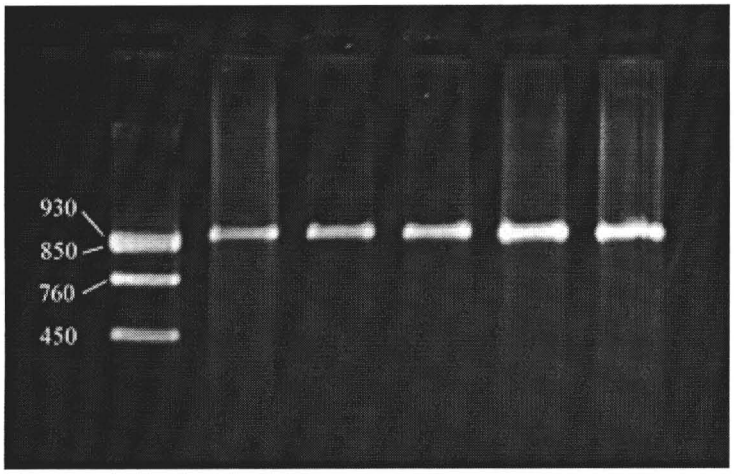


Figure 3.3. PCR amplification of target region. Molecular marker is p-Bluescript KS cut with *Pvu* II and *Dra* I.

C. Saturation

Multiple mutations at a single nucleotide site, termed overprinting or saturation, reduces the amount of signal in sequence data. The cytochrome b gene has limited utility in answering broad scale phylogenetic questions due to overprinting between distantly related taxa (see Chapter One; Graybeal 1993; Meyer 1994; Griffiths 1997). Even though this study investigates intraspecific variation, saturation is still a consideration.

As found in most sequence data, the majority of mutations occured in the third codon position and transitions outnumber transversions 3:1 (Fitch 1967). The relationship of transitions to transversions is further discussed in section 3.2.D. Although there are 10 amino acid changes within *L. hochstetteri*, there is only one overprinted base (table 3.2), which indicates a clear phylogenetic signal exists within the species. Much more variation emerges when *L. archeyi* is included, and there is an increase in the number of overprinted bases (table 3.2). However, since *L. archeyi* is closest extant sister taxa to *L. hochstetteri* (Hay *et al.* 1995; Holyoake *et al.* 1999, in press), there is no ~~better choice~~ for an outgroup.

Table 3.2. Mutation analysis The number and type of mutation is given for the *L. hochstetteri* sequences alone, and for the *L. hochstetteri* and *L. archeyi* sequences combined. Each type of mutation is presented according to codon position. Data format is as follows: total number of mutations (transitions, transversions, multiple hits). For the protein data, the number of amino acid changes is given.

Data	*1st Position*	*2nd position*	*3rd Position*	*Protein*
L. hochstetteri	13(11,2,0)	4(3,1,0)	37(29,7,1)	10
All sequences	31(21,8,2)	9(8,1,0)	110(74,28,8)	26

D. Models of sequence evolution

Many different phylogenetic models attempt to correct inaccuracies in genetic analyses caused by the processes of sequence evolution (for a review see Chapter 6 of Page and Holmes 1998). In the absence of a corrective model, sequence analysis works on the assumption that each kind of mutation, whether A to T or A to G or C, is equally likely to occur. This assumption fails in most instances because natural sequences have a certain amount of base pair distortion and transitions are more likely to occur than transversions (Fitch 1967).

In this study, the HKY85 model was used to compensate for these irregularities (Hasegawa, Kishimo and Yano 1985). The HKY85 model accounts for differences in nucleotide frequencies as well as differences in the number of transitions versus transversion (Hasegawa, Kishimo and Yano 1985). The values used to make these corrections were estimated from the data. The mean frequencies of each type of nucleotide were A=.27619, C=.26673, G=.14784, and T=.30925. These nucleotide frequencies were consistent across the entire data set (X^2=7.042, df=81, p=1.0). The transition/transversion ratio was calculated using the method described in Page & Holmes (1998) in which the log likelihoods of heuristic trees constructed using different transition/transversion ratios are compared to determine the ratio that has the greatest likelihood of producing the data. For this data set, the best transition/transversion ratio is 3:1 (figure 3.4).

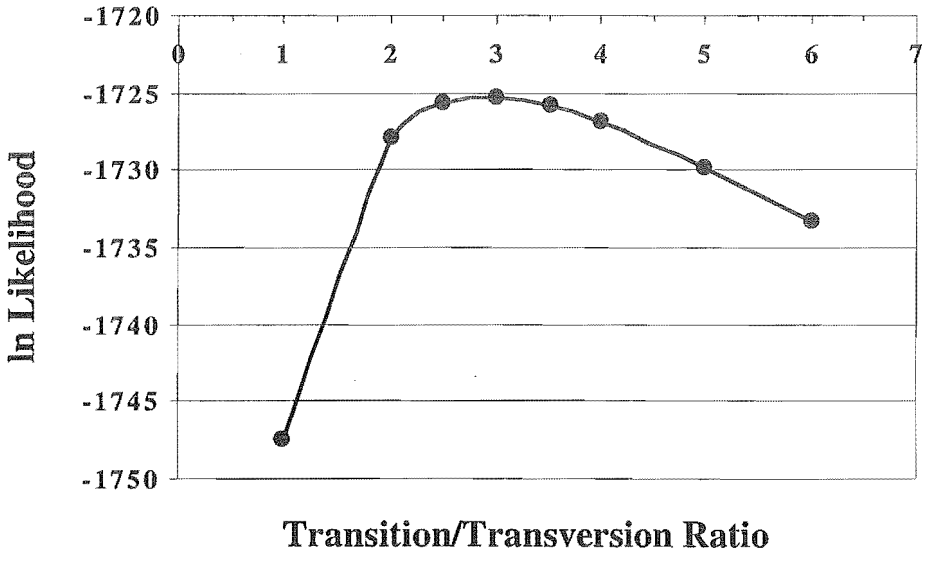


Figure 3.4. Calculation of transtition/transversion ratio The correct transition/transversion ratio is that which maximizes the likelihood of producing the data. Since the curve peaks at 3:1, that is the optimum ratio for this data set

phylogenetic ?

E. Translation to amino acid sequence

When conducting DNA sequence analysis, many assumptions are made about the processes of sequence evolution, such as those described above (see Chapter 5 in Page and Holmes 1998). Although these assumptions are supported by a great body of research, they are still assumptions. To eliminate assumptions about codon biases and

transition/transversion ratios, the protein sequence can be used for phylogenetic analysis instead of the nucleotide sequence. Protein sequences are a more conservative source of information on phylogenetic relationships since silent mutations, which could be saturated, are eliminated from the data.

To translate the *Leiopelma* cytochrome b nucleotide sequence to amino acid data, the reading frame and genetic code from *Xenopus laevis* mtDNA was used (Roe 1985). The amino sequence for the different haplotypes appears in appendix D.

F. Rooting

Rooting is an important part of sequence analysis because the outgroup provides polarity to the characters and establishes the direction of sequence evolution. Choosing the right outgroup is critical. Outgroups that are too distantly related to the ingroup will cause long branch attraction between the outgroup and the longest branch in the ingroup (Smith 1994). When long branch attraction occurs, the longest branch in the ingroup is paired with the outgroup not because of true relatedness, but because overprinting between the lineages causes the two taxa to be groups by chance (Hendy and Penny 1989; Wheeler 1990; Smith, Lafay and Christen 1992).

To limit the effects of long branch attraction and ensure the correct polarisation of characters, Smith (1994) advises using the sister taxa to the ingroup as the outgroup. This method works especially well if more than one individual from the sister taxa is represented. To account for these considerations individuals from two populations of *L. archeyi* were used as outgroups in this study.

3.3 Reconstructing Molecular Phylogenies

Many methods exist for creating trees of relationship from sequence data, the most popular of which are genetic distance (Sneath and Sokal 1973), parsimony (Edwards and Cavalli-Sforza 1963; Camin and Sokal 1965) and maximum likelihood (Felsenstein 1981). Distance methods use pairwise genetic distances, such as percent sequence differences, between taxa to build a tree (Sneath and Sokal 1973). This type of analysis is especially useful when analysing data from DNA hybridization studies where the relationship between taxa is already expressed by a number, but tend to oversimplify genetic data by eliminating useful information such as the type and location of particular mutations (Page and Holmes 1998). When constructing a tree, the distance algorithm groups together taxa that have the smallest distances between them. Although genetic distance is an index of relatedness, a tree based on genetic distances does not identify historical or ancestral relationships between taxa but only the amount of sequence change. Since most evolutionary studies are concerned with ancestral relationships between taxa, genetic distance methods are of limited use. Distance methods for building trees were not used in this study because the historical relationships between different populations was of interest. Also, sequence variation between individuals was not great and therefore information on the types of mutations was too valuable to lose (Page and Holmes 1998).

Parsimony and maximum likelihood are both discrete methods operating on the sequences themselves. The basis of parsimony is the assumption that the tree which requires the fewest number of changes to account for the differences between taxa is the best tree (Edwards and Cavalli-Sforza 1963; Camin and Sokal 1965). When parsimony is applied to sequence data, each nucleotide base is evaluated to find the most parsimonious arrangement. A tree which represents base number 124 changing from an A to C once will be favoured over the tree which has the same base changing from C to A back to C. Each type of genetic mutation can be weighted as more or less "costly" using the models of sequence evolution described in section 3.2.D.

Parsimony is a popular and widely used method of phylogenetic reconstruction but there is a drawback. Parsimony has been known to produce incorrect phylogenies in instances of long branch attraction (see section 3.2.F), when overprinting obscures the ancestral relationships between long branches and causes taxa to be incorrectly paired (Hendy and Penny 1989). For parsimony to recover the correct tree under such circumstances, there must be more changes supporting the correct tree than any of the other possible relationship. This may not be the case due to homoplasy (Hendy and Penny 1989).

Maximum likelihood (Felsenstein 1981) works in the opposite direction to parsimony. Parsimony seeks to determine the most probable tree given the data while maximum likelihood determines how likely it would be to see the observed data given a particular tree (Felsenstein 1981). The goal of maximum likelihood is to find the tree which makes the data the most probable evolutionary outcome given a particular model of sequence evolutions (see section 3.2.D)(Felsenstein 1981). Maximum likelihood performs better than parsimony in situations involving long branches because it accounts for both the branch lengths and ancestral state of each node (Page and Holmes 1998). If the two branches leaving a node are long, maximum likelihood places less weight on the ancestral state at that node being shared between the two taxa. If nucleotide change is rare, parsimony and maximum likelihood tend to give the same tree (Felsenstein 1973). If that assumption is relaxed, parsimony and maximum likelihood diverge (Felsenstein 1973).

The in-depth analysis which maximum likelihood provides is also the source of its greatest drawback. Maximum likelihood is computationally expensive, especially for large data sets. However, this is less of a limitation as computers and phylogenetic software become more powerful. I chose to use the maximum likelihood method of phylogenetic reconstruction for this study because it has been consistently demonstrated to produce the more robust phylogenetic tree compared with other available methods (Page and Holmes 1998).

3.4 Confidence Limits for Phylogenies

Even with an accurate model of sequence evolution and an efficient method of phylogenetic reconstruction, any given phylogenetic tree is only one representation of the evolutionary relationship. It is important to be able to distinguish between alternative

hypotheses. Often, more than one "best" tree exists, each with different branching patterns. The presence of more than one optimal tree can result from unresolvable interior nodes. Most phylogenetic methods assume that nodes bifurcate. When this assumption is invalid, statistical support for internal nodes can be very low. An unresolved node may represent either an absence of enough data to resolve the node or an instance of a radiation (Maddison 1989; De Salle *et al.* 1994; Hoelzer and Melnick 1994). Two commonly employed methods for placing confidence limits on trees are bootstrapping (Felsenstein 1985) and quartet puzzling (Strimmer and von Haeseler, 1996).

Bootstrapping identifies the statistical support for each node by pseudoreplication (Felsenstein 1985). The method samples nucleotide sites from the data set with replacement, until the number of sites sampled is the same as in the original data set. Then, a tree is created using the pseudoreplicated data set. This process is repeated at least 100 times, ~~but often more~~. The frequency with which a particular node appears in the data set represents the statistical support for that node. Nodes with a support of 50% or over are generally considered significant, although greater than 95% was originally recommended by Felsenstein (1985).

Bootstrapping is widely used, but there are some drawbacks to this approach (Sanderson 1995; Sitnikova, Rzhetsky and Nei 1995). Bootstrapping is a conservative method which usually underestimates the statistical support for a node when compared to other methods (Sitnikova, Rzhetsky and Nei 1995). Also, bootstrapping tends to become increasingly more conservative as the number of taxa in the data set increases (Sitnikova, Rzhetsky and Nei 1995).

Unlike bootstrapping which uses pseudoreplication, quartet puzzling uses quartet trees to determine support for particular nodes (Strimmer and von Haeseler, 1996). All possible four taxon trees from a data set are evaluated using maximum likelihood criteria to find the best quartet for each possible combination. Taxa are then added sequentially to an intermediate tree. The ~~taxa's~~ location in each quartet determines its final placement in the intermediate tree. Once all taxa have been added, the intermediate tree is stored and the process begins again. There is no one tree which best reflects all quartets, so the process is repeated approximately 1000 times to find as many optimal trees as possible. In the final step a majority- rule consensus tree is drawn based on all optimal trees. The number of times a node appears in an intermediate tree determines its confidence value in the final consensus tree.

Quartet puzzling does not use pseudoreplication, so the entire data set is represented in each tree. This is a consideration because pseudoreplication during bootstrapping might underrepresent variation in a data set (Sanderson 1995). Given that the sequence variation is already low between populations of the same species, quartet puzzling was preferred for this study. Another advantage to quartet puzzling was its speed in PAUP* 4.0 (Swofford 1998), which was significantly faster than the bootstrap test.

3.5 Phylogeny of *L. hochstetteri*

The phylogeny of *L. hochstetteri* based on sequence information from part of the cytochrome b gene can be found in figure 3.5. The tree was constructed using maximum likelihood criterion with quartet puzzling. The tree based on the protein sequence is found in figure 3.6, and was constructed using parsimony criteria because maximum likelihood criteria cannot be applied to protein data in PAUP* 4.0 (Swofford 1998). Sequences from *L. archeyi* were stipulated as the outgroup for both trees.

The implications these tree have for the phylogenetic history of *L. hochstetteri* is discussed in Chapter 4. This allowed for comparisons between phylogenetic tree building methods and other way of investigating the same relationships.

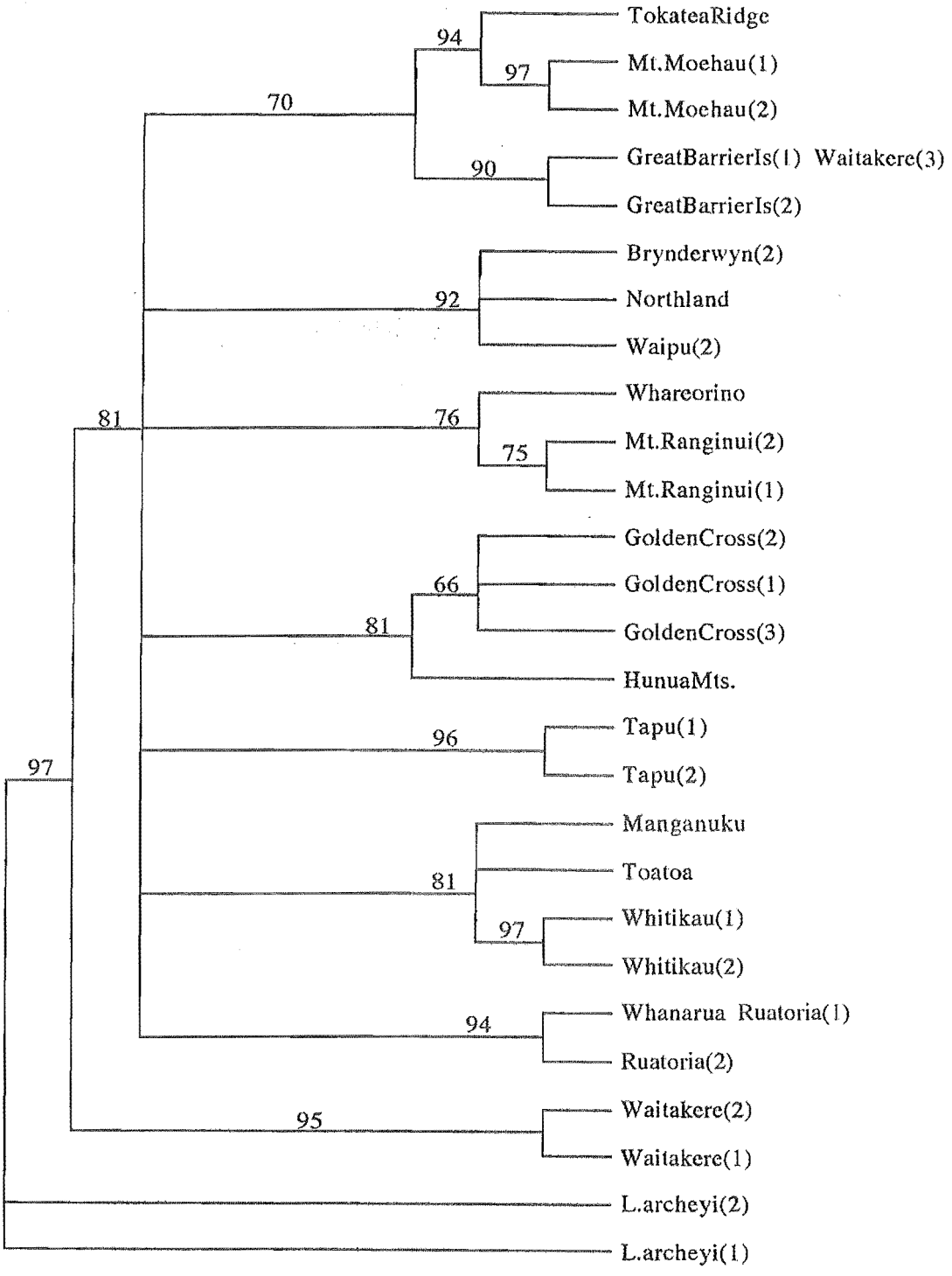


Figure 3.5 Phylogenetic relationships within *L. hochstetteri*: sequence data A maximum likelihood puzzle tree with *L. archeyi* as the outgroup. The confidence values appear above each node. Numbers to the right of the site names designate each haplotype found in that location. The number of individuals represented by each haplotype appears in appendix B. Nodes with a confidence value less than 60 were collapsed. The homoplasy index equals 0.149 and the consistency index is 0.851.

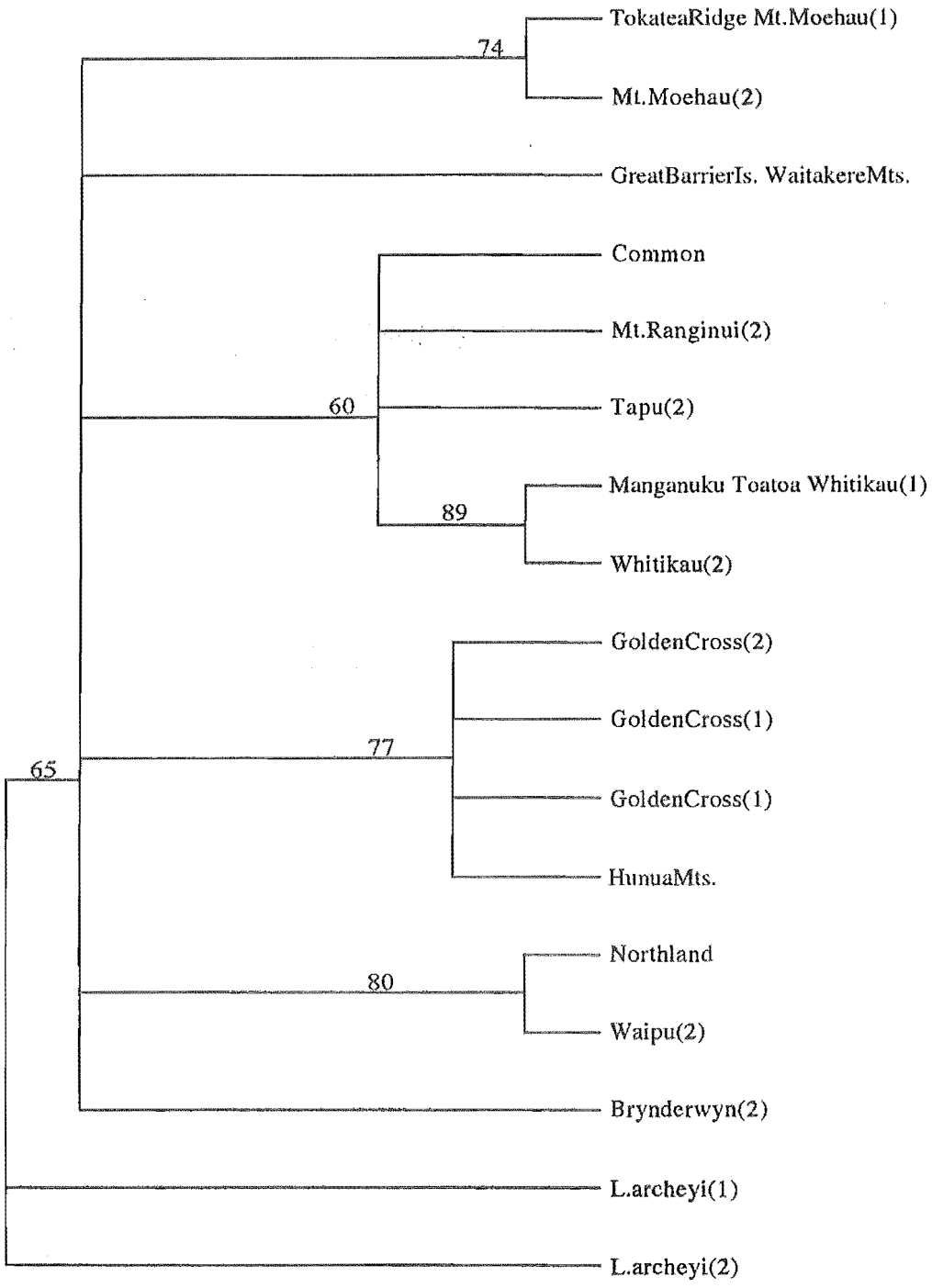


Figure 3.6 Phylogenetic relationships within *L. hochstetteri*: protein data A parsimony tree with quartet puzzling. *L. archeyi* was stipulated as the outgroup. Confidence values appear above each node. Numbers to the right of the site names designate the mitochondrial haplotypes of the original sequence data. A list of the mitochondrial haplotypes found in the "Common" protein haplotype appears in appendix D.

Chapter Four

Analysis of Population Structure

This chapter investigates the population structure of *Leiopelma hochstetteri* using a minimum spanning network and analysis of molecular variance (AMOVA). Minimum spanning networks avoid the constraints of bifurcation and enable tree topologies to include alternative branches. Analysis of molecular variance is a statistical approach used to investigate genetic variation at different hierarchical levels and proved valuable for detecting populations structure. The last section in the chapter summarises all the information gleaned on the phylogeny and population structure of *L. hochstetteri* from the various phylogenetic methods previously described in Chapters Three and Four.

4.1 Minimum Spanning Network

A bifurcating tree is not always the most effective framework for presenting phylogenetic data. As discussed in Chapter One, unresolved nodes and homoplasy can cause misleading relationships or even errors in a bifurcating tree (Hendy and Penny 1989; Hoelzer and Melnick 1994). These difficulties stem from the presence of more than one equally plausible branching pattern (Maddison 1989). A tree with many branching possibilities is best represented by a network because each haplotype can be connected to as many other haplotypes as is necessary (Excoffier and Smouse 1994).

Prim (1975) designed the general algorithm for minimum spanning networks, but the idea has been adapted by others for use with molecular data (Excoffier and Smouse 1994). Minimum spanning networks differ from other phylogenetic trees in that haplotypes are represented as internal nodes and not the ends of branches. In a network, a single node can be connected to many others, an option which is impossible in a bifurcating phylogeny. The basic algorithm for creating a minimum spanning network (Excoffier and Smouse 1994) has four steps: (1) Start with N unconnected nodes (haplotypes). Take any node A, and find a node B whose distance from A is shortest and link node A to node B. This connection forms a subtree. (2) Find unconnected node C closest to a member of the subtree, say A, and connect A to C. Connect all unconnected nodes equally close to that member of the subtree to which C is connected, and connect C to all equally closest nodes of the spanning subtree. (3) Repeat step (2) until all nodes are connected.

The algorithm uses a distance matrix to determine the length of each branch as well as the branching pattern. Any type of distance matrix can be used to draw the network, but the program used in this study (Minspnet, Excoffier and Smouse 1994) requires a matrix of pairwise nucleotide differences. The distance matrix was calculated using PAUP* 4.0

(Swofford 1998) and can be found in appendix E. The program Minspnet (Excoffier and Smouse 1994) determined the connections between nodes. The network was drawn by hand using the branching pattern provided by the program. The minimum spanning network is presented in figure 4.1.

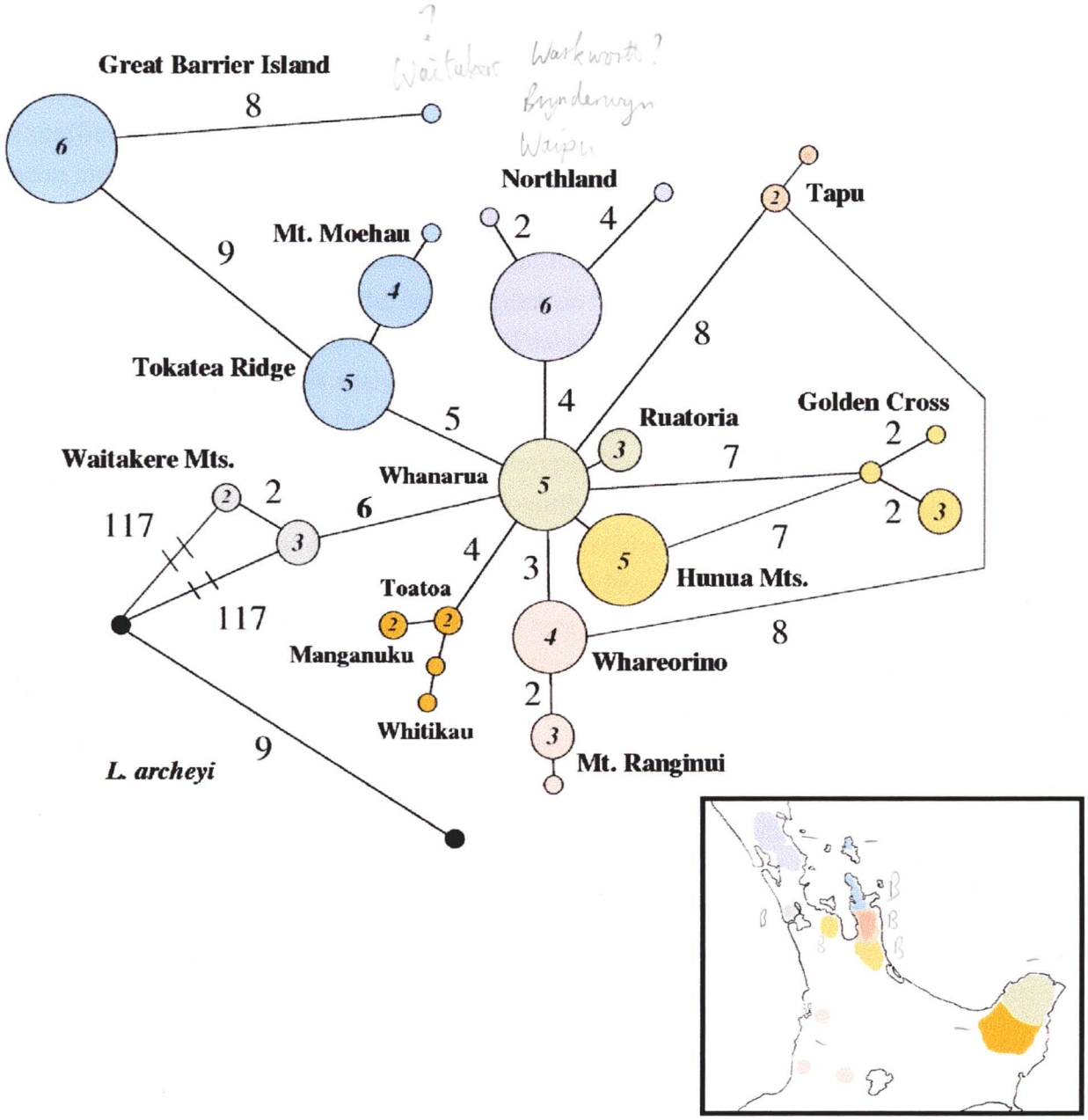


Figure 4.1 Minimum spanning network depicting the relationship between populations of *L. hochstetteri* and *L.archeyi*. The length of the branches denotes the number of nucleotide changes between haplotypes. Branches of one nucleotide difference are not labelled. Numbers inside the circles designate the number of individuals displaying that haplotype. When only one individual is represented by a haplotype, that circle is unlabeled. The colours correspond to the regions on the colour-coded map.

4.2 Analysis of Molecular Variance

Analysis of Molecular Variance (AMOVA) is a common method of testing the amount of population structure at different hierarchical levels (Excoffier *et al.* 1992). This study utilised the AMOVA analysis employed in Genetic Analysis in Excel (GenAlEx, Peakall and Smouse 1998). This program calculated the pairwise genetic distances between haploid sequence data according to Huff *et al.* (1993), and performed the analysis of molecular variance as described by Excoffier *et al.* (1992). Three different hierarchical levels were investigated: the variation between individuals within a population, the variation between populations within a region, and the variation between regions. For each level a Φ-statistic, analogous to Wright's F-statistic (Wright 1965), was calculated. The significance level of the Φ-statistic was calculated using 999 iterations of the null distribution for each variance component, considered sufficient for publication purposes (Peakall and Smouse 1998).

For the purposes of this analysis, each haplotype was treated as an individual. Populations were defined as all the haplotypes from a particular sampling site, with the exception of sampling sites which shared a haplotype. These exceptions included the haplotypes from Brynderwyn, Waipu and Warkworth which were all considered one population, and Whanarua and Ruatoria which also shared a haplotype. All populations were grouped into three geographical areas, the Coromandel Peninsula and Great Barrier Island, the East Cape Peninsula, and the Auckland/Waikato area.

The hierarchial analysis enabled the investigation of genetic variance in the species as a whole, and the separate analysis of the three geographical areas. By analysing each area separately, the level of genetic structure could be broken down further to include other hierarchical levels. For example, the Coromandel Peninsula was divided into two regions with a border above Tapu to test whether there was significant genetic structure between north and south. East Cape was also divided into north and south regions with the division between Toatoa and Ruatoria. The Auckland/Waikato area was divided in the Auckland Peninsula-Northland region and Waikato. An alternative division of the Auckland/Waikato area was also investigated in which the Waitakere Mts. were considered a separate region from the rest of the populations. The results of these AMOVAs can be found in table 4.1.

Table 4.1 Analysis of Molecular Variance percent genetic variation, the Φ-statistic and p-value for each hierarchy level is given. The p-value is based on a comparison to the null distribution.

Data set	*Individuals within populations*			*Among populations/ within regions*			*Among Regions*		
	% var.	Φ	P	% var.	Φ	P	% var.	Φ	P
All *L. hochstetteri*	23	0.773	0.001	64	0.740	0.001	13	0.127	0.001
Coromandel	26	0.739	0.001	51	0.660	0.001	23	0.232	0.071
East Cape	22	0.779	0.033	13	0.377	0.193	65	0.645	0.060
Auckland/Waikato	20	0.803	0.001	78	0.799	0.001	2	0.385	0.022
Auckland/Waitakere	15	0.850	0.001	40	0.729	0.001	45	0.446	0.023

4.3 Significant genetic structure in *L. hochstetteri*

Throughout its range, *L. hochstetteri* is highly structured at the population level (64%, Φ = 0.740, p = 0.001). When the populations are grouped into three geographical areas, Coromandel, East Cape, and Auckland/Waikato, there is little significant genetic structure between areas (13%, Φ = 0.127, p = 0.001). The lack of regional structure is further supported by the large basal polytomy in the quartet puzzling tree (figure 3.5). Whereas the clades defining populations have high confidence values (figure 3.5) the basal nodes are unresolved and show no significant correlation with geographic locality. Given this lack of phylogenetic resolution, it would not be appropriate to consider these geographic areas as phylogenetic groups. However, each geographic area does have defining characteristics in its population structure which are worthy of in-depth discussion.

4.4 Regional genetic diversity

A. Coromandel Peninsula

Within the Coromandel there is no significant structure above the population level. Although 26% of the variation appears within populations (Φ = 0.739, p = 0.001), over half the variation is found between populations (51%, Φ = 0.660, p = 0.001). The populations are genetically disparate and there are few clear relationships between them. The Mt. Moehau and Tokatea Ridge populations are the only two that can be grouped with a high degree of certainty. This relationship is supported by the maximum likelihood quartet puzzling tree (confidence value = 95, figure 3.5) and also appears in the minimum spanning network (figure 4.1). The minimum spanning network also shows the Mt. Moehau-Tokatea ridge clade as the closest relative to the Great Barrier Island population (confidence value = 70, figure 3.5). There is a lot of genetic difference (9 nucleotide changes) between Great Barrier Is and Tokatea Ridge (figure 4.1). This is not surprising considering Great Barrier Is. has been

completely isolated from the North Island populations since the sea level rose approximately 10,000 years ago following the last glaciation (Flemming 1975).

Both Tapu and Golden Cross are genetically distinct from each other, and the rest of the populations on the Coromandel. Golden Cross contains a great amount of within population diversity with three out of the five individuals displaying a different haplotype, all of which are distinct protein haplotypes.

B. East Cape

Although there is a clear geographic distinction between sampling sites in the north and south of the East Cape peninsula, the AMOVA showed no significant genetic differentiation between those two areas (65%, $\Phi = 0.645$, $p = 0.060$), but the differentiation was close to significant. A distinction between populations in the north and south of East Cape is supported by the quartet puzzling tree (figure 3.5), and the minimum spanning network (figure 4.1). Although there is very little variation between individuals (22%, $\Phi = 0.779$, $p = 0.033$) or populations (13%, $\Phi = 0.377$, $p = 0.193$) in both regions, there are four nucleotide changes between the north and south and no shared haplotypes (figure 4.1). These four changes result in amino acid differences in the protein sequence, suggesting the populations have been isolated for some time (figure 3.6).

Two haplotypes are present in the Ruatoria population. The Ruatoria frog were collected from two streams only a five-minute drive from each other (see appendix B). The two haplotypes segregate between the streams, and one of the haplotypes is shared with the Whanarua population. There appears to be no gene flow between these two Ruatoria streams since the haplotypes segregate between them. Gene flow between the Ruatoria stream with the shared haplotype and the more distant Wharanrua stream can be inferred. Such a fine scale of population substructure is a glimpse of the detail that could be gained from more extensive sampling.

C. Auckland Peninsula and Waikato

Unlike the Coromandel Peninsula and East Cape, the populations in the Auckland Peninsula and the Waikato do not form one continuous distribution, but are a series of isolated populations. There were no *a priori* groups within these isolated populations, so the Auckland Peninsula and Waikato were grouped together for the purposes of the AMOVA. There was no significant genetic distinction between the Auckland Peninsula and Waikato (2%, $\Phi = 0.022$, $p = 0.385$), which is not surprising considering 78% of the variation occurs between populations in both regions ($\Phi = 0.799$, $p = 0.001$). Such a great amount of genetic structure at the population level indicates the populations are highly genetically isolated from each other.

Within the Auckland/Waikato area, the only populations occupying a continuous distribution are the three populations from the Northland region. Brynderwyn, Waipu and

Warkworth all shared a common haplotype, indicating the existence of significant gene flow between these populations either now or in the past. Brynderwyn and Waipu each contain one haplotype unique to those populations in addition to the shared haplotype. However, the majority of frogs in all three populations displayed the shared haplotype, so these three localities were considered one population for the purposes of the AMOVA.

The relationship of the Waitakere Mts. population to the rest of the Auckland/Waikato area is inconclusive. The quartet puzzling tree places the Waitakere Mts. as basal to the rest of the populations (confidence value = 97, figure 3.5). The minimum spanning network also places the Waitakere Mts. as most closely related to *L. archeyi* (figure 4.1). If we define two regions in the AMOVA, one being the Waitakere populations and the other the rest of the Auckland/Waikato area, there is significant genetic variance between the two (45%, Φ = 0.446, p = 0.023). This evidence indicates the Waitakere population is genetically distinct from other *L. hochstetteri*. However, this distinction is not as definite as it initially appears. Frogs from the Waitkere Mts. are grouped basaly to the rest of *L. hochstetteri* in the quartet puzzling tree based on mtDNA sequence (figure 3.5), but when translated to protein these frogs have the same amino acid sequence as Great Barrier Is. (figure 3.6). If *L. archeyi* is removed from the analysis, the unrooted tree places the Waitkere Mts as basal to the Great Barrier-Tokatea Ridge-Mt. Moehau clade (confidence value = 91, figure 4.2).

The sequence difference between *L. archeyi* and *L. hochstetteri* (20%) is high for an outgroup-ingroup relationship. The separation of the Waitakere Mts. from all other populations could be the result of long branch attraction (see section 3.2.C and 3.2.F), and the unrooted tree of *L. hochstetteri* supports this conclusion (figure 4.2). Homoplasy between *L. archeyi* and the Waitakere Mts. population would also cause the two taxa to be connected in the minimum spanning tree because the Waitakere Mts. would have the smallest pairwise distance to *L. archeyi*. However the homoplasy index for the maximum likelihood puzzle tree is low (0.149, figure 3.5) and is almost identical to the homoplasy index of the same tree with *L. archeyi* removed (0.154, figure 4.2). A low homoplasy index for both trees indicates that long branch attraction might be less of a problem than initially thought.

Although the Hunua Mts lie on the Auckland peninsula, they are phylogenetically grouped with Golden Cross in the quartet puzzling tree (confidence level = 81, figure 3.5) and the protein tree (confidence value = 77, figure 3.6). This close association suggests that these populations were continuous in the recent past, which is not surprising considering their distribution would be adjoining if the Hunua Mts. population extended farther south. The Whareorino and Mt. Ranginui populations in the Waikato constitute a significant grouping in the quartet puzzling tree (confidence value = 76, figure 3.6). However, there is no significant regional structure when these Waitkato populations are compared to the Auckland Peninsula populations (2%, Φ = 0.385, p = 0.022).

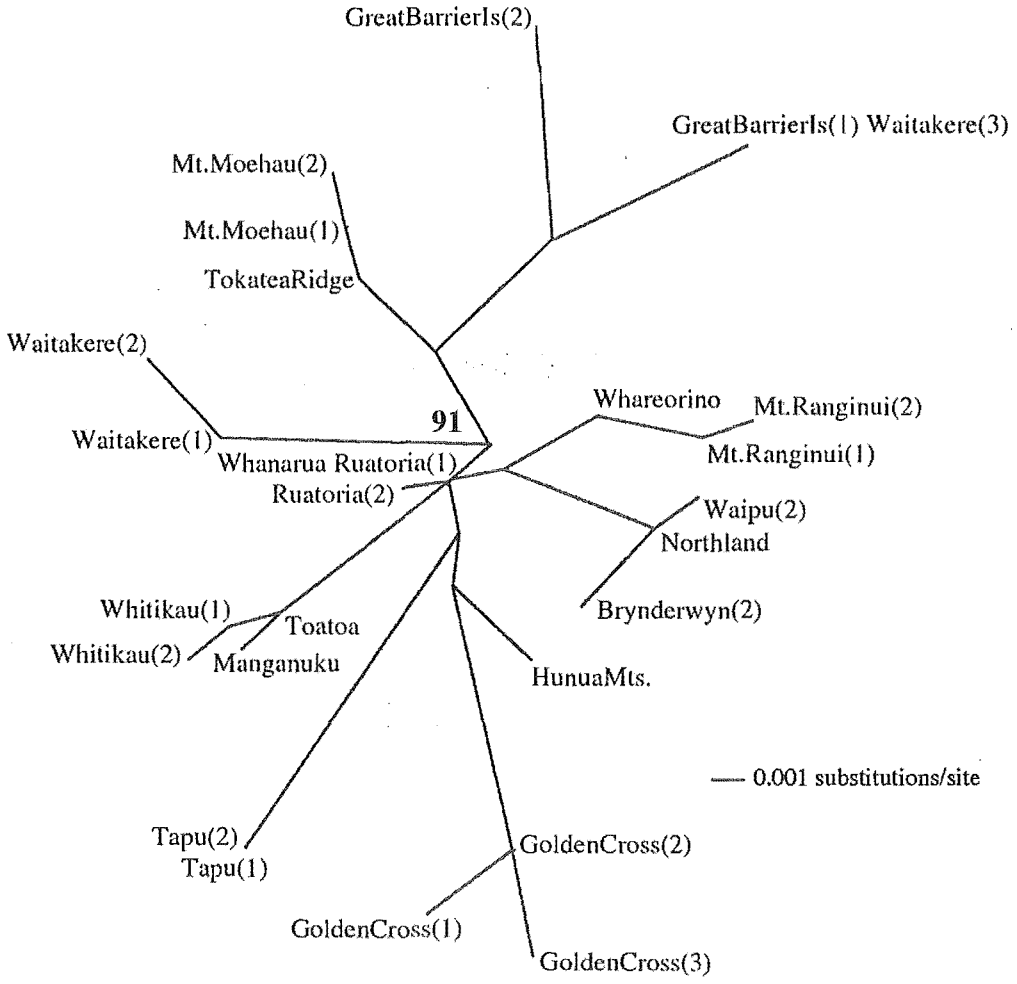


Figure 4.2 Unrooted maximum likelihood tree depicting relationships between populations of *L. hochstetteri*. Numbers to the right of location names denote the mitochondrial haplotypes of the original sequence data. The quartet puzzling confidence value for the node designating the relationship between the Waitakere Mts. and the northern Coromandel is in bold face. The homoplasy index equals 0.154 and the consistency index is 0.846.

Chapter Five

Chromosome Change and Genetic Change

This chapter investigates the relationship between B-chromosome numbers and the mt DNA based phylogeny of *L. hochstetteri*. The segregation of chromosome numbers within haplotypes, as well as the correlation between chromosome number and population variation is investigated.

5.1 B-Chromosomes and phylogeny in *L. hochstetteri*

In Chapter One, the potential affects of B-chromosomes as agents of speciation were discussed (see section 1.5). To determine what affect, if any, B-chromosomes have on the genetic structure of *L. hochstetteri* the distribution of B-chromosomes within and among populations must be investigated. The B-chromosome number of each frog appears in appendix B, courtesy of D. M. Green. For the purposes of this analysis, individuals with unknown karyotypes were excluded.

Figure 5.1 shows the number of B-chromosomes found in every individual within each haplotype alongside the corresponding phylogeny of those haplotypes. The B-chromosome numbers in figure 5.1 do not include the univalent sex chromosome, which is found in all females except those from Great Barrier Island. The phylogenetic relationships between populations containing B-chromosomes reveal information on the chromosome's origin and historical inheritance. In a phylogeny, basal taxa are thought to possess the ancestral character state with interior branches exhibiting the derived state (Page and Holmes 1998). Taxa which occupy a single clade and share a derived character state inherited from a common ancestor are considered monophyletic (Freeman and Herron 1998). The shared character state has a single origin within the clade. B-chromosomes occur on many branches of the tree (figure 5.1), and do not demonstrate any signs of monophly. In figure 5.1, the Waitakere populations are basal to the rest of *L. hochstetteri*, with the exception of haplotype three. According to this relationship, having B-chromosomes is the ancestral state of *L. hochstetteri* because that is the state found in the basal taxa. However, the basal position of the Waitakere population could be an artefact of long branch attraction (see section 4.4.C). Since the position of the Waitakere population is ambiguous, the B-chromosome number in other members of the genus must be assumed to be ancestral. *L. archeyi* does not have any B-chromosomes (Green 1988b), and *L. hamiltoni* and *L. pakeka* only have a univalent sex chromosome (Green 1988). Based on chromosome data from sister taxa, the absence of B-chromosomes is the ancestral state of *L. hochstetteri*.

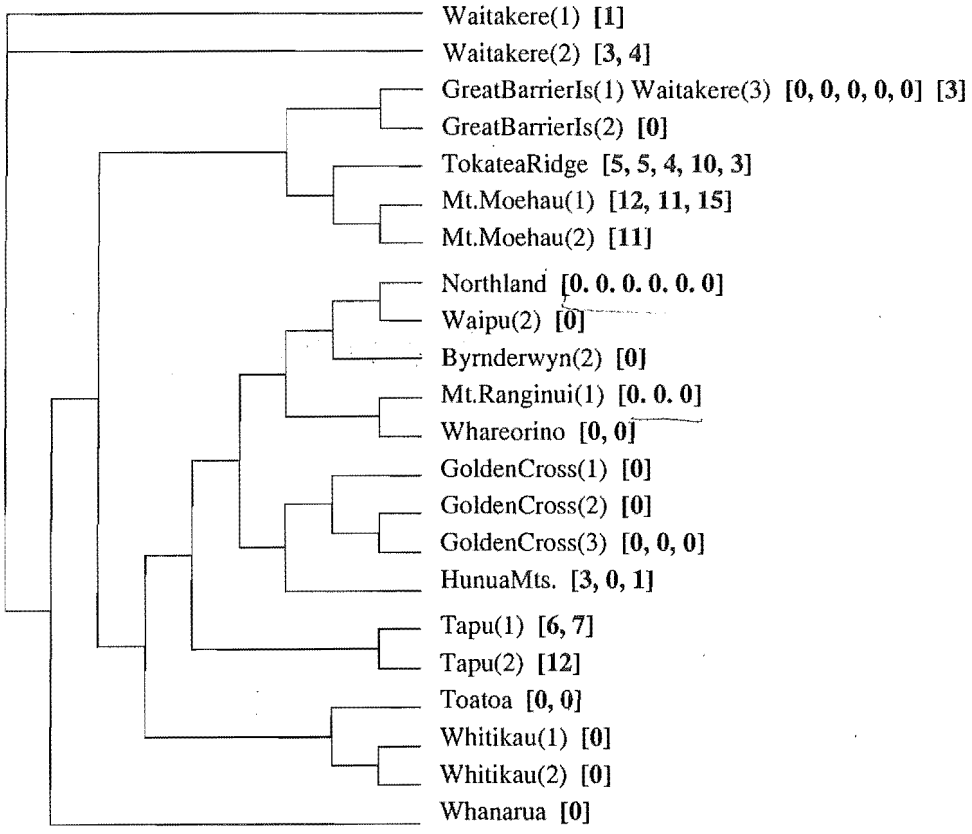


Figure 5.1 B-chromosomes in *L. hochstetteri* The tree was constructed using maximum likelihood criterion with quartet puzzling. Numbers beside haplotype names indicate the number of B-chromosomes found in individuals of that haplotype. B-chromosome numbers do not include the univalent sex chromosome. Individuals with unknown karyotype were excluded. No outgroup was stipulated.

Taxa which exhibit the same character, but did not inherit it from a common ancestor, are termed polyphyletic, and the character is thought to have multiple origins (Freeman and Herron 1998). Since the absence of B-chromosomes is the ancestral state and haplotypes with B-chromosomes are polyphyletic, B-chromosomes evolved multiple times in *L. hochstetteri*. According to the phylogeny, there are three to four separate chromosomal lineages, depending on the phlyogenetic relationship of the Waitakere Mts. population. Four separate B-chromosome lineages appear in figure 5.1. If the Waitakere population really belongs in the same clade as Mt. Moehau and Toakatea ridge (see section 4.4.C), then the B-chromosomes of the Waitakere population are part of the same chromosomal lineage. The other B-chromosome lineages that evolved independently are those in Tapu and the Hunua Mountains.

In the clade containing the Hunua Mts., not all populations within the clade exhibit B-chromosomes. Golden Cross appears in the same clade as the Hunua Mts., but does not have B-chromosomes. The Hunua Mts. population is basal in relation to Golden Cross, indicating their common ancestor had B-chromosomes. Presumably, Golden Cross once had B-chromosomes, but they were lost through drift or selection.

The phylogeny in figure 4.2 suggests the same could be true of Great Barrier Island. If the Waitakere population is basal to the clade containing Great Barrier Island (figure 4.2), then the ancestral population of Great Barrier Island would have had B-chromosomes.

5.2 B-chromosomes and population variation

B-chromosomes are thought to be responsible for reproductive isolation (John 1973, 1981; Green 1991; McVean 1998). If B-chromosomes have an isolating effect in *L. hochstetteri*, then that isolation would appear in the phylogeny in two ways. Reduced fecundity of mating pairs with highly divergent chromosome numbers would drive the segregation of haplotypes by favouring matings between individuals of similar chromosome number. Therefore, chromosome number should segregate according to haplotype. The second effect of B-chromosomes would appear in the number of haplotypes in populations with B-chromosomes. If B-chromosomes cause reduced fertility in mating pairs with different chromosome numbers, then populations with variable numbers of B-chromosomes should contain more haplotypes.

Segregation of B-chromosome number between haplotypes can only be investigated in populations with both B-chromosomes and more than one haplotype. Unfortunately, these two requirements only apply to three populations, the Waitkaere Mts., Mt. Moehau, and Tapu populations. In both the Waitakere Mts. and Mt. Moehau, the number of B-chromosomes in each haplotype is roughly equal. However, haplotype number two in Tapu has twice the number of B-chromosomes as haplotype number one. Unfortunately, only three individuals were sequenced from the Tapu population.

To test whether B-chromosomes increase population genetic diversity, the average number of haplotypes per population was calculated for populations with and without B-chromosomes. The mean number of haplotypes for populations without B-chromosomes was 1.6 ± 0.49, and the mean for populations with B-chromosomes was 1.8 ± 0.70. Because the standard deviations of these means overlap, the means are not significantly different from each other.

The ramifications of these results for both *L. hochstetteri* and the study of B-chromosomes in general are discussed in section 6.3 of the next chapter.

Chapter Six

Discussion

This chapter concludes the discoveries made in this thesis. The phylogeny of *L. hochstetteri* is discussed in relation to the evolutionary history of the species, and is used to examine the origin and evolution of B-chromosomes in this species. The influences of B-chromosomes on the populations structure are also discussed. Both genetic and karyological data are used to make suggestions concerning the conservation management of *L. hochstetteri*. Finally, potential areas for future research on *L. hochstetteri* are presented.

6.1 Phylogenetic relationships within *Leiopelma*

As has been concluded in other studies using morphology and allozymes (Daugherty 1981; Green 1989) and sequence variation (Holyoake *et al.*, in press), *L. hochstetteri* shows high molecular divergence from other *Leiopelma* species. Green (1989) has argued that *L. hochstetteri* should be placed in a separate genus, a proposition which is supported by the results of this study. Approximately 20% sequence difference exist between *L. archeyi* and *L. hochstetteri*, such a level of difference is unusual for an intra-generic relationship (Green 1989). Although two *L. archeyi* samples are not enough to draw phylogenetic conclusions, this amount of sequence difference may result in long branch attraction (see section 3.2.C and 3.2.F). There is evidence of long branch attraction in this study (see section 5.2.C), but no alternative outgroup exists as *L. archeyi* is the most closely related extant sister species (Holyoake *et al.* 1999, in press).

6.2 Biogeography of L. hochstetteri

Studies on *L. hochstetteri* tend to use the isolation of Great Barrier Island from the rest of the North Island as a reference point when describing the history of the species (Green 1989; Green *et al.* 1993). Green *et al.* (1993) argues that the univalent sex chromosome appeared in the species before the isolation of Great Barrier Island, and after the isolation the other B-chromosomes evolved in the North Island populations. Although this argument makes sense in light of chromosomal evidence, mtDNA sequence evidence shows that the phylogenetic relationships within *L. hochstetteri* do not necessarily support this hypothesis.

During the Otiran glaciation (2-5 mya, Suggate 1990), the isolated North Island peninsulas that *L. hochstetteri* currently inhabits were connected by landmasses (Flemming 1975). As the sea level rose between 14,000 to 8,000 ya, the North Island peninsulas formed and Great Barrier Island separated from the rest of the North Island (Flemming 1975). The maximum likelihood quartet puzzling tree based on sequence variation (figure 3.5) shows Great Barrier Island populations diverging from the rest of the northern

Coromandel populations after the large radiation of all other clades. Such a relationship indicates that *L. hochstetteri* populations were already genetically isolated before sea level rise separated the peninsulas of the North Island. Evidence from the AMOVA also supports this hypothesis because little of the genetic variance in the species is distributed within peninsular areas (13%, $\Phi = 0.127$, $p = 0.001$), but is found primarily at the population level (64%, $\Phi = 0.740$, $p = 0.001$).

As has been demonstrated by the AMOVA, *L. hochstetteri* exhibits highly localised genetic variation. Instead of investigating genetic structure from a broad geographic perspective, a more localised investigation of topography is warranted. It is possible that local geography had a much stronger impact on the genetic structure of *L. hochstetteri* than post-glacial sea level rise. Highly localised genetic structure has also been found in other anuran species (Hitchings and Beebee 1997; Hanken 1999), indicating fine scale population isolation is not unique to *L. hochstetteri*.

The Coromandel is an area of extreme population differentiation for *L. hochstetteri*. The Coromandel is also mountainous and individual catchment can be separated by dry elevated land. This geography might heavily constrain dispersal of *L. hochstetteri* because these frogs are dependent on streams for their survival (Tessier *et al.* 1991). Consequently, little gene flow between populations would be expected. In contrast, the Northland region is flat and moist compared to the Coromandel, so gene flow between populations is more likely. This argument is supported by the fact that the three populations in Northland share a common haplotype, suggesting current or recent genetic exchange.

The evolutionary history of each haplotype is unresolved in figure 4.1 due to the presence of a large basal polytomy. The minimum spanning network may describe the historical relationships between haplotypes more effectively. Although there is no way to determine the original mitochondrial ancestor of *L. hochstetteri*, the haplotype at Whanarua is the centre of the network for all other haplotypes (figure 4.1). At least two interpretations of this relationship exist. Possibly, Whanarua is the ancestral haplotype from which all populations radiated. Alternatively, the Whanarua haplotype is the point of radiation for other haplotypes but is a few steps away from the ancestral state, which exists on one of the terminal branches. Considering the current geography of the North Island, Whanarua as the centre of radiation for *L. hochstetteri* seems unlikely. Whanarua is located on the very edge of the species' distribution (figure 1.2), and different colonisations from this remote location would be required to produce genetically disparate population in places like the Coromandel. Although Tapu and Golden Cross are geographically adjacent, their haplotypes would have originated from two separate colonisations from the Whanarua haplotype. An alternative explanation for the placement of Whanarua in the centre of the network is that the Whanarua haplotype was historically found across the North Island. Other populations have evolved along their own branches, while the Whanarua population has retained the ancestral haplotype.

In Chapter Three, the limitations of phenetic methods of phylogenetic reconstruction were discussed, including the fact that distance methods do not designate evolutionary relationships between taxa (Sneath and Sokal 1973, see section 3.3). This limitation is true of the minimum spanning network because the network is based purely

on the number of nucleotide changes between haplotypes and does not take into account the ancestral states of those nucleotides. However, the phenetic nature of the network does not nullify its significance. The maximum likelihood tree in figure 4.2 shares many visual aspects with the minimum spanning network because it is an unrooted phylogram. Unlike the network, the maximum likelihood tree accounts for the ancestry of each character (Felsenstein 1981). Despite the fact that these two trees are based on different tree building methods, their topologies are very similar and support the same relationships between populations, thereby suggesting these relationships are significant.

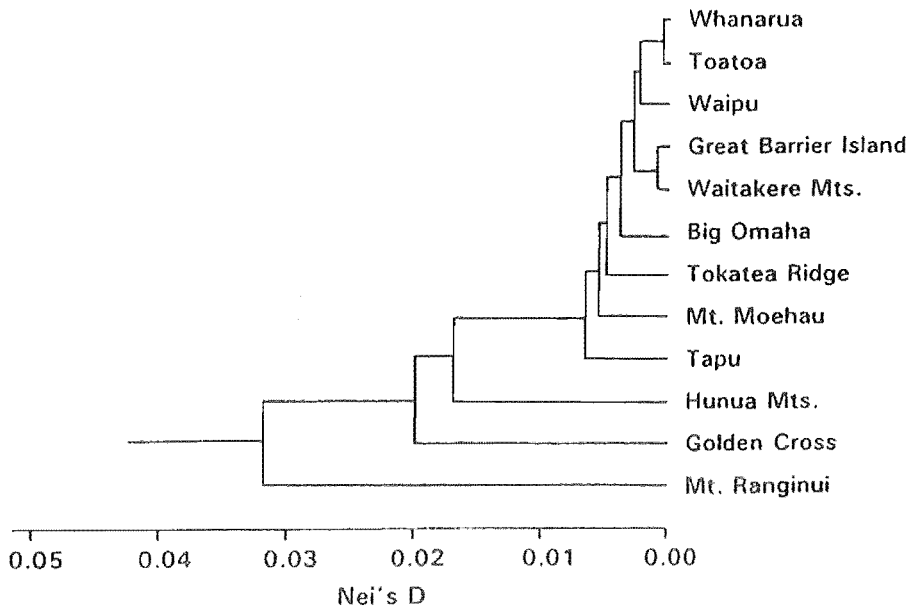


Figure 6.1 Phylogeny of *L. hochstetteri* using allozyme data. Rooted Fitch-Margoliash tree for Nei's *D* for 41 allozyme loci. Figure from Green (1994).

Green (1994) constructed a phylogenetic tree representing the relationship between different populations of *L. hochstetteri* using allozyme data. Green's (1994) tree is reproduced in figure 6.1. The tree is phenetic and therefore does not describe ancestral relationships, but only the genetic distance between populations. Figure 6.1 differs significantly from any trees produced in this study. According to allozyme data, the most divergent populations are Mt. Ranginui, Golden Cross, and the Hunua Mountains. Although the Golden Cross population is highly divergent according to sequence data (figure 4.2), the other populations do not show a large amount of divergence from the central radiation. Populations that are highly divergent with respect to sequence data (figure 4.2), do not exhibit the same degree of divergence based on allozymes (figure 6.1), such as the Waitakere Mts., Great Barrier Island and Tapu. The strongest similarity between the two trees is the grouping of the Waitakere Mts. population with Great Barrier Island population. Waitakere haplotype number three is identical to Great Barrier Island haplotype one in figure 3.5, and the protein sequences for all Great Barrier Island and Waitakere haplotypes are identical (figure 3.6). The lack of correlation between figure 6.1 and the molecular evidence from this study could be explained by the small amount of

variation in allozyme loci between populations (Green 1994). Very small genetic difference between nodes in figure 6.1 may mean the nodes are unresolvable, and the relationships between them, as displayed, are arbitrary. Therefore, it is not surprising that the phylogeny based on allozyme data collected by Green (1994) does not correlate with the genetic relationships found in this study.

6.3 Chromosome variation in *L. hochstetteri*

In Chapter Five (section 5.2), two hypothesises were presented which described the potential effects of B-chromosomes on population genetic structure. If B-chromosomes cause reduced fertility in matings between individuals with different chromosome numbers, then populations with variable numbers of B-chromosomes should contain more haplotypes. In addition, reduced fertility between mating pairs with different chromosome numbers would promote the segregation of B-chromosome number by haplotype.

This study found no evidence that suggests that B-chromosomes affect fertility in *L. hochstetteri*. An increase in the number of B-chromosomes did not correlate with an increase in the number of haplotypes in a population, nor did B-chromosomes segregate between haplotypes. Green *et al.* (1993) has argued for a recent origin of all B-chromosomes. Due to their absence on Great Barrier Island, Green *et al.* (1993) hypothesises that B-chromosomes have evolved after the separation of Great Barrier Island from the North Island approximately 10,000 years ago (Flemming 1975). Ten thousand years is not a long time in an evolutionary sense, so it is possible that the B-chromosomes' effect on reproductive isolation has not had time to appear in these molecular data. However, if B-chromosomes had any impact on fertility, some amount of lineage sorting would be present.

Sharbel *et al.* (1998) have argued for a single origin of B-chromosomes from the univalent sex chromosome, based on sequence similarity between B-chromosomes in different populations (see section 1.4). However, sequence data from the cytochrome b gene support more than one origin of B-chromosomes (see section 5.1). It is possible that there were as many as four independent origins for these B-chromosomes: the Waitakere Mts. lineage, the Tapu lineage, the Hunua Mts. lineage and the Mt. Moehau-Tokatea Ridge lineage. Furthermore, the phylogeny of the species (figure 5.1) suggests that the B-chromosomes have been gained but lost on at least one occasion. B-chromosomes are observed in the Hunua Mts population but have been lost in the Golden Cross population. This observation is consistent with the characterisation of B-chromosomes as selfish genetic elements (Green 1991). Once the univalent sex chromosome was fixed in North Island populations, it would have been possible to have more than one evolution of B-chromosomes since they tend to propagate themselves (White 1973; Green 1991; McVean 1998), and can even reappear in populations which have lost them (King 1981). Although Sharbel *et al.* (1998) support the hypothesis of a single origin for B-chromosomes, they admit to possibility of multiple origins based on B-chromosome morphology.

According to the phylogeny in figure 3.5, the possibility exists that some of the chromosomal lineages appeared before Great Barrier Island was separated from the North

Island. The clades representing the Tapu and Hunua Mts. chromosomal lineages originated at the basal polytomy, before the Great Barrier Island population separated from the northern Coromandel. If gene flow between populations had virtually ceased before 10 000 ya, as is suggested in section 6.2, then the migration of B-chromosomes to different localities would be unlikely. The phylogenetic evidence suggest the evolution of B-chromosomes in *L. hochstetteri* occurred earlier than previously thought (Green *et al.* 1993)

6.4 Implications for conservation

From a conservation standpoint, *L. hochstetteri* has received much less attention than its other sister taxa, especially *L. pakeka* and *L. hamiltoni* (Bell *et al.* 1998). Although the number of *L. pakeka* and *L. hamiltoni* are much lower than that of *L. hochstetteri* (Bell 1985), both of these species live on island reserves where they are monitored, and introduced predators are absent (Newman 1996). In contrast, not all Conservancies monitor populations of *L. hochstetteri*, and populations which are located outside of reserves are severely threatened by introduced species and commercial development (Newman 1996).

L. pakeka and *L. hamiltoni* are nearly identical for 300 bps of the cytochrome b gene (Holyoake *et al.* 1999, in press). Within *L. hochstetteri* there was as much as 3% sequence difference in 600bps of the same gene. Although it would be impractical and unfounded to split *L. hochstetteri* into more than one species, it is important to note that populations of this species need to be considered as separate evolutionary groups.

One method of species' management uses the identification of ESUs (Evolutionarily Significant Units) to define genetically important populations for conservation (see section 1.1). When evaluating populations of *L. hochstetteri*, the task is fairly easy. Since most of the genetic variation in this species resides at the population level (63%, phi = 0.720, p = 0.001), virtually every population of *L. hochstetteri* in this study could be viewed as an ESU. Of course, such a conclusion is not entirely practical and is similar to arguing that saving the species requires the preservation of every population. This is not entirely true since some populations can be justifiably grouped into larger ESUs.

The East Cape peninsula is a region containing little genetic variation based on this study and that of Green (1994). There is only one nucleotide difference between populations besides a change of four base pairs between the north and south (figure 4.1). The whole peninsula could be considered an ESU, and the loss of one population within the region may not be detrimental to the genetic identity of the species. The other ESUs would follow the significant clades identified by the maximum likelihood puzzle tree (figure 3.5). Tokatea Ridge and Mt. Mochau would form one ESU, and Great Barrier Island would be separate. Tapu would be one ESU, and Golden Cross would be grouped with the Hunua Mts.. Northland (including Brynderwyn, Waipu and Warkworth) would constitute another group, as would the Waitakere Mountains. Whareorino and Mt. Ranginui would comprise a single ESU.

In the Native Frog Recovery Plan section 8.0 (Newman 1996), the prioritisation of different populations for conservation is discussed. Although the hierarchy includes populations of other native frogs as well as *L. hochstetteri*, populations of *L. hochstetteri* are prioritised in the following order:

1. **Whareorino**
2. Great Barrier Island
3. Waipu
4. Rangitoto Range (Mt. Ranginui)
5. **South Coromandel (Golden Cross)**
6. East Cape
7. Warkworth
8. **Central Coromandel (Tapu)**
9. **North Coromandel (Mt. Moehau and Tokatea Ridge)**
10. Hunua Mts.
11. Waitakere Mts.

The locations in boldface also contain populations of *L. archeyi* and therefore have been prioritised with that species in mind as well. The significance of those locations with respect to *L. archeyi* cannot be commented on in this study. However, in terms of *L. hochstetteri*, the reprioritisation of some populations should be considered. Based on both B-chromosome and sequence evidence, this study supports prioritisation in the following order:

1. Great Barrier Island
2. Waitakere Mts.
3. Central Coromandel (Tapu)
4. South Coromandel (Golden Cross)
5. North Coromandel (Mt. Moehau and Tokatea Ridge)
6. Northland
7. East Cape
8. Whareorino and Mt. Ranginui

The Great Barrier Island population is unique in its B-chromosome composition, and is genetically divergent from even its closest relatives in the northern Coromandel (figure 4.1). The Waitakere Mts. were ranked second due to their ambiguous phylogenetic relationship (see section 4.4.C), and the possibility that they could constitute a basal clade to the rest of the species (figure 3.5). Although the Coromandel populations are ranked third to fifth, the regions have almost equal importance. Tapu is both genetically divergent and chromosomally variable, as are Mt. Moehau and Tokatea Ridge. Golden Cross is genetically divergent and is important because it lacks the B-chromosomes found in neighbouring populations. In the Native Frog Recovery Plan, Waipu was given high priority because extensive mining in the area was causing habitat degradation (Newman

1996). Given that evidence of recent gene flow exists between all Northland populations sampled in this study, frogs in Waipu could be transferred to other habitat in the neighbouring areas without jeopardising the genetic identity of the species. The populations in Northland, East Cape and the Waikato are not exceptional in terms of B-chromosomes or sequence variation.

6.5 Future research

Hopefully, this research will promote the popularity of *L. hochstetteri* as a species worthy of further study, since it has received less attention than other native frog species (cite). Although this study was a thorough investigation of sequence variation in the cytochrome b gene, it is only the tip of an iceberg of potential molecular studies. Comparing the data from cytochrome b with other genes is a logical first step. It has been widely acknowledged that as the number of genes increase, so does the resolution of the phylogeny (Avise and Wollenburg 1998). The use of a gene with a high mutation rate, such as an intron or other non-coding region, could resolve the polytomy at the base of the *L. hochstetteri* clade and elucidate the relationships between populations (Avise and Wollenburg 1998). Better phylogenetic resolution could also be achieved with more extensive sampling, particularly in areas between genetically disparate populations. The example of the southern half of East Cape, where four slightly different haplotypes exist in a small area, demonstrates the fine scale resolution which can be achieved with extensive sampling (see section 4.4.B).

The affect of B-chromosomes on population structure requires more investigation. As a contrast to a molecular enquiry, a mating study could examine the reproductive relationships between chromosome races more directly. Mating studies could also examine the relationship between genetic distance and reproductive isolation, a question that has important implications for the study of speciation (Dobzhansky 1935; Mayr 1963).

6.6 Conclusions

Leiopelma hochstetteri is highly structured at the population level. This structure was established before the end of the last glaciation and was probably caused by local topography rather than sea level rise, which has been implicated in the formation of chromosome variation (Green *et al.* 1993).

B-chromosomes have had no visible affect on the establishment of genetic structure in *L. hochstetteri*. No evidence from the study suggests B-chromosomes have an affect on reproductive isolation between populations. The phylogeny indicates B-chromosomes have arisen many times within the species, and were possibly present before the end of the last glaciation. These conclusions differ from those of previous studies, which argued for a single origin of B-chromosomes from the univalent sex chromosome (Sharbel *et al.* 1998) sometime after the end of the Otiran glaciation (Green *et al.* 1993). However, this study does support the work of many who have found B-chromosomes to be highly variable and enigmatic karyological elements (White 1973; Robinson and Roux 1985; Green 1991).

Although this study attempts to answer many questions concerning the genetic history and populations structure of *L. hochstetteri*, the species deserves more attention. There is much that remains unknown about the behaviour and ecology of this frog. To preserve this species from extinction requires knowledge on all aspects of its biology.

Appendix A

Table A.1 Solutions. All solutions taken from Sambrook, *et al.* (1989)

Solution	*Composition*
Digestion Solution	5% Chelex
	100mM NaCl
	50mM Tris pH8.0
	10mM EDTA
Gel-loading Buffer	0.25% bromophenol blue
	0.25% xylene cyanol FF
	30% glycerol in water
TE-Chelex	5% Chelex
	10mM Tris pH8.0
	1mM EDTA
TBE	0.045M Tris-borate
	0.001M EDTA
TE pH8.0	10mM Tris-Cl pH8.0
	1mM EDTA pH8.0

Appendix B

B.1 Origin of DNA samples

Species	*Location*	*Sample number*	*Sex*	*Number of B-chromosomes (2n=22+X)*
Leiopelma archeyi	Golden Cross[1]	0400	-	-
	Whareorino[2]	0105	-	-
Leiopelma hochstetteri	Brynderwyn	3005	F	1
		3006	M	0
		3007	F	1
	Golden Cross	3029	F	1
		3030	M	0
		4224	F	1
		4225	M	0
		4226	F	1
	Great Barrier Island	2999	F	0
		3000	F	0
		3001	F	0
		3002	M	0
		3031	F	0
		3032	F	0
	Hunua Mountains	2121	F	4
		2122	M	0
		2152	F	2
		3024	M	-
		3025	F	-
	Manganuku[3]	1005	-	-
		1006	-	-
	Mt. Moehau	2124	F	13
		2208	F	12
		2995	F	12
		5132	M	15
		5186	F	-
	Mt. Ranginui	3034	F	1
		3050	M	-
		3056	F	1
		3064	F	1
	Ruatoria	1030	-	-

	1050	-	-
	1053	-	-
	1054	-	-
Tapu	2109	F	7
	2210	F	6
	2230	F	13
Toatoa	2103	F	1
	3490	F	1
Tokatea Ridge	2108	F	6
	2112	M	5
	2117	F	5
	2272	F	4
	3779	-	-
Waipu	3035	F	1
	3036	M	0
	3048	M	0
Waitakere Mountains	3018	F	2
	3020	F	4
	3021	F	5
	3022	F	4
	3037	F	-
	5127	F	-
Warkworth	2143	M	0
	2144	F	1
Whanarua	3075	F	1
Whanarua[3]	1011	-	-
	1013	-	-
	1015	-	-
Whareorino	5183	M	0
	5185	M	0
Whareorino[2]	27	-	-
	55	-	-
Whitikau	2146	M	0
	2213	M	0

[1]Samples collected by B. Waldman
[2]Samples collected by K. Eggers
[3]Samples collected by the author and N. J. Gemmell
All other samples collected by D. M. Green

B.2 Collection of samples from East Cape

Little Manganuku River

GPS coordinates: NZMS 260 X16 (28938, 63156). Seven samples were collected from small tributaries of the Little Manganuku River, located on the Department of Conservation "Little Manganuku Trail". Frogs were found in two tributaries approximately 2000 meters apart.

Whanarua Stream

GPS coordinates: NZMS 260 Y14 (29322, 63795). Six samples were collected from tributaries of the Whanarua Stream.

Ruatoria State Forest (Ruakumera)

Twelve samples were collected from the same stream in a pine plantation with almost 25 years worth of growth. The density of frogs there was especially high compared to other streams. Five more samples were collected from the next stream over, approximately a five-minute drive from the previous stream.

Appendix C

The complete sequences of the 27 mitochondrial DNA haplotypes used in this study. The haplotype number appears in brackets after the location name. Ambiguities in the nucleotide sequence are denoted by "N." The number of individuals included in each haplotype is as follows:

L.archeyi(1)	1	GoldenCross(1)	1
L.archeyi(2)	1	GoldenCross(2)	1
Waitakere(1)	3	GoldenCross(3)	3
Waitakere(2)	2	Mt.Ranginui(1)	3
GreatBarrierIs(1) Waitakere(3)	6	Mt.Ranginui(2)	1
GreatBarrierIs(2)	1	Whareorino	4
TokateaRidge	5	HunuaMts.	5
Mt.Moehau(1)	4	Manganuku	2
Mt.Moehau(2)	1	Toatoa	2
Northland	6	Whitikau(1)	1
Brynerwyn(2)	1	Whitikau(2)	1
Waipu(2)	1	Whanarua Ruatoria(1)	5
Tapu(1)	2	Ruatoria(2)	3
Tapu(2)	1		

```
[                                    10        20        30        40        50]
[                                    .         .         .         .         .]
L.archeyi(1)                         TCACACACATCTGCCGAGATGTCAACTGCGGATGACTAATCCGAAATATG
L.archeyi(2)                         ..G.........................................
Waitakere(1)                         ..........T.....G..C..T....A...G...T............C
Waitakere(2)                         ..........T.....G..C..T....A...G...T............C
GreatBarrierIs(1) Waitakere(3)       .......T..T.....G..C..T....A...G...T............C
GreatBarrierIs(2)                    .......T..T.....G..C..T....A...G...T............C
TokateaRidge                         .......T..T.....G..C..T....A...G...T............C
Mt.Moehau(1)                         .......T..T.....G..C..T....A...G...T............C
Mt.Moehau(2)                         ....N..T..T.....G..C..T....A...G...T............C
Northland                            .......T..T.....G..C..T....A...G...T............C
Brynderwyn(2)                        .......T..T.....G..C..T....A...G...T............C
Waipu(2)                             .......T..T.....G..C..T....A...G...T............C
Tapu(1)                              .......T..T.....G..C..T....A.......T............C
Tapu(2)                              .......T..T.....G..C..T....A.......T............C
GoldenCross(1)                       ..G....T..T.....G..C..T....A...G...T............C
GoldenCross(2)                       ..G...NT..T.....G..C..T....A...G...T............C
GoldenCross(3)                       ..G....T..T.....G..C..T....A...G...T............C
Mt.Ranginui(1)                       .......T..T.....G..C..T....A...G...T............C
Mt.Ranginui(2)                       .......T..T.....G..C..T....A...G...T............C
Whareorino                           .......T..T.....G..C..T....A...G...T............C
HunuaMts.                            ..G....T..T.....G..C..T....A...G...T............C
Manganuku                            .......T..T.....G..C..T....A...G..GT............C
Toatoa                               .......T..T.....G..C..T....A...G..GT............C
Whitikau(1)                          ......NT..T.....G..C..T....A......GT............C
Whitikau(2)                          .......T..T.....G..C..T..T.AN.....GT............C
Whanarua Ruatoria(1)                 .......T..T.....G..C..T....A...G...T............C
Ruatoria(2)                          .......T..T.....G..C..T....A...G...T............C
```

```
[                                        60         70         80         90        100]
[                                        .          .          .          .         .]
L.archeyi(1)                             CATGCCAACGGGGCCTCACTTTTCTTCATTTGCATTTACCTGCACATCGG
L.archeyi(2)                             ...........A................................
Waitakere(1)                             ...........A..T..TT.A..T........T.....TT.A..T.....
Waitakere(2)                             ...........A..T..TT.A..T..............TT.A..T.....
GreatBarrierIs(1) Waitakere(3)           ...........A..T..TT.A..T........T.....TT.A..T..T..
GreatBarrierIs(2)                        ...........A..T..TT.A..T..T.....T.....TT.A..T..T..
TokateaRidge                             ...........A..T..TT.A..T........T.....TT.A..T..T..
Mt.Moehau(1)                             ........T..A..T..TT.A..T........T.....TT.A..T..T..
Mt.Moehau(2)                             ......N.T..A..T..TT.A..T........T.....TT.A..T..T..
Northland                                ...........A..T..TT.A..T........T.....TT.A..T.....
Brynderwyn(2)                            ...........A..T..TT.A..T........T.....TT.A..T.....
Waipu(2)                                 ...........A..T..TT.A..T........T.....TT.A..T.....
Tapu(1)                                  ...........A..T..TT.A..T........T.....TT.A..T.....
Tapu(2)                                  ...........A..T..TTNA..T........T.....TT.A..T.....
GoldenCross(1)                           ...........A..T..TT.A..T........T.....TT.A..T.....
GoldenCross(2)                           ...........A..T..TT.A..T........T.....TT.A..T.....
GoldenCross(3)                           ...........A..T..TT.A..T........T.....TT.A..T.....
Mt.Ranginui(1)                           ...........A..T..TT.A..T........T.....TT.A..T.....
Mt.Ranginui(2)                           ......NT...A..T..TT.A..T........T.....TT.A..T.....
Whareorino                               ...........A..T..TT.A..T........T.....TT.A..T.....
HunuaMts.                                ...........A..T..TT.A..T........T.....T..A..T.....
Manganuku                                ...........A..T..TT.A..T........T.....TT.A..T.....
Toatoa                                   ...........A..T..TT.A..T........T.....TT.A..T.....
Whitikau(1)                              ...........A..T..TT.A..T........T.....TT.A..T.....
Whitikau(2)                              ...........A..T..TT.A..T........T.....TT.A..T.....
Whanarua Ruatoria(1)                     ...........A..T..TT.A..T........T.....TT.A..T.....
Ruatoria(2)                              ...........A..T..TT.A..T........T.....TT.A..T.....
```

(V) (V)

```
[                                       110        120        130        140        150]
[                                       .          .          .          .         .]
L.archeyi(1)                            ACGCGGCATGTACTACGGATCTTACCTGTTCAAAGAAACATGAAATATCG
L.archeyi(2)                            ..................................................
Waitakere(1)                            ...A.....A..........C.....A.....................T.
Waitakere(2)                            ...A.....A..........C.....A.....................T.
GreatBarrierIs(1) Waitakere(3)          ...A.....A..............A.....................T.
GreatBarrierIs(2)                       ...A.....A..............A.....................T.
TokateaRidge                            ...A.....A..........C.....A.....................T.
Mt.Moehau(1)                            ...A.....A..........C.....A.....................T.
Mt.Moehau(2)                            ...A.....A..........C.....A.....................T.
Northland                               ...A.....A..........C.....A.....................T.
Brynderwyn(2)                           ...A.....A..........C.....A.....................T.
Waipu(2)                                ...A.....A..........C.....A.....................T.
Tapu(1)                                 ...G.....A..........C.....A.....................T.
Tapu(2)                                 ...G.....A..........C.....A.....................T.
GoldenCross(1)                          ...A.....A..........C.....A.....................T.
GoldenCross(2)                          ...A.....A..........C.....A.....................T.
GoldenCross(3)                          ...A.....A..........C.....A.....................T.
Mt.Ranginui(1)                          ...A.....A..........C.....A.....................T.
Mt.Ranginui(2)                          ...A.....A..........C.....A.....................T.
Whareorino                              ...A.....A..........C.....A.....................T.
HunuaMts.                               ...A.....A..........C.....A................G...T.
Manganuku                               ...A.....A..........C.....A.....................T.
Toatoa                                  ...A.....A..........C.....A.....................T.
Whitikau(1)                             ...A.....A..........C.....A.....................T.
Whitikau(2)                             ...A.....A..........C.....A.....................T.
Whanarua_Ruatoria(1)                    ...A.....A..........C.....A.....................T.
Ruatoria(2)                             ...A.....A..........C.....T.....................T.
```

(V) V

```
[                                        160        170        180        190        200]
[                                        .          .          .          .          .]
L.archeyi(1)                             GCGTCGTCCTATTATTTCTAGTTATAGCAACAGCCTTTGTAGGCTATGTT
L.archeyi(2)                             .....................C.........................
Waitakere(1)                             ....ANN...T.............................C........C
Waitakere(2)                             ....ANN...T.............................C........C
GreatBarrierIs(1) Waitakere(3)           ....ANN...T.....T.......................C........C
GreatBarrierIs(2)                        ....ANN...T.....T.......................C........C
TokateaRidge                             ....ANN...T.....T.......................C........C
Mt.Moehau(1)                             ....ANN...T.....T.......................C........C
Mt.Moehau(2)                             ....ANN...T.....T.......................C........C
Northland                                ....ANN...T.....T.......................C........C
Brynderwyn(2)                            ....ANN...T.....T.......................C........C
Waipu(2)                                 ....ANNN..T.....T.......................C........C
Tapu(1)                                  ....ANN...T.....T.......................C........C
Tapu(2)                                  ....ANNN..T.....T.......................C........C
GoldenCross(1)                           ....ANN...T.....T.......................C........C
GoldenCross(2)                           ....ANNN..T.....T.......................C........C
GoldenCross(3)                           ....ANN...T.....T.......................C........C
Mt.Ranginui(1)                           ....ANN...T.....T.......................C........C
Mt.Ranginui(2)                           ....ANN...T.....T.......................C........C
Whareorino                               ....ANN...T.....T.......................C........C
HunuaMts.                                ....ANN...T.....T.......................C........C
Manganuku                                ....ANN...T.....T.........................
Toatoa                                   ....ANN...T.....T.......................C........C
Whitikau(1)                              ....ANN...T.....T.......................C........C
Whitikau(2)                              ....ANN...T.....T.......................C........C
Whanarua_Ruatoria(1)                     ....ANN...T.....T.......................C........C
Ruatoria(2)                              ....ANN...T.....T.......................C........C
```

(V) V

```
[                                        210        220        230        240        250]
[                                        .          .          .          .          .]
L.archeyi(1)                             CTGCCATGAGGGCAGATATCCTTCTGAGGCGCTACAGTAATTACTAATCT
L.archeyi(2)                             .................................................
Waitakere(1)                             T.A.....G..A..A.....T..T.....G..C.....T..C.....C..
Waitakere(2)                             T.A.....G..A..A.....T..T.....G..C.....T..C.....C..
GreatBarrierIs(1) Waitakere(3)           T.A..C.....A..A..G..T..T.....A..C.....T..C.....C..
GreatBarrierIs(2)                        T.A..C..G..A..A..G..T..T.....G..C.....T..C.....C..
TokateaRidge                             T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Mt.Moehau(1)                             T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Mt.Moehau(2)                             T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Northland                                T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Brynderwyn(2)                            T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Waipu(2)                                 T.A.....G..A..A..G.NT..T.....G..C.....T..C.....C..
Tapu(1)                                  T.A........A..A..G..T..T.....G..C.....T..C.....C..
Tapu(2)                                  T.A........A..A..G..T..T.....G..C.....T..C.....C..
GoldenCross(1)                           ..A.....G..A..A..GN.T..T.....A..C.....T..C.....C..
GoldenCross(2)                           ..A.....G..A..A..GN.T..T.....A..C.....T..C.....C..
GoldenCross(3)                           ..A.....G..A..A..G..T..T.....A..C.....T..C.....C..
Mt.Ranginui(1)                           T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Mt.Ranginui(2)                           T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Whareorino                               T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
HunuaMts.                                T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Manganuku                                T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Toatoa                                   T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Whitikau(1)                              T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Whitikau(2)                              T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Whanarua_Ruatoria(1)                     T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
Ruatoria(2)                              T.A.....G..A..A..G..T..T.....G..C.....T..C.....C..
```

V (V)

```
[                                    260        270        280        290        300]
[                                    .          .          .          .          .]
L.archeyi(1)                         CCTCTCTGCTATCCCGTATGTCGGAAATACAATAGTACAATGAATTTGAG
L.archeyi(2)                         ..............C..............................
Waitakere(1)                         TT.A..A..C..A..A...A....G.GC...C....T.............
Waitakere(2)                         TT.A..A..C..A..A...A....G.GC...C....T.............
GreatBarrierIs(1) Waitakere(3)       TT.A..A..C..G..A...A.T..G.GC...C....T...........G.
GreatBarrierIs(2)                    TT.A..A..C..A..A...A.T..G.GC...C....T...........G.
TokateaRidge                         TT.A..A..C..A..A...A.T..GGGC...C....T.............
Mt.Moehau(1)                         TT.A..A..C..A..A...A.T..GGGC...C....T.............
Mt.Moehau(2)                         TT.A..A..C..A..A...A.T..GGGC...C....T.............
Northland                            TT.A..A..C..A..A...A....GGGC...C....T.............
Brynderwyn(2)                        TT.A..A..C..A..A...A....GGGC...C....T.............
Waipu(2)                             TT.A..A..C..A..A...A....GGGC...C....T.............
Tapu(1)                              TT.A..A..C..A..A...A....G.GC..GC....T.............
Tapu(2)                              TT.A..A..C..A..A...A....G.GC..GC....T.............
GoldenCross(1)                       TT.A..A..C..A..A...A....GGGC...C....T.............
GoldenCross(2)                       TT.A..A..C..A..A...A....GGGC...C....T.............
GoldenCross(3)                       TT.A..A..C.CA..A...A....GG.C...C....T.............
Mt.Ranginui(1)                       TT.A..A..C..A..A...A....G.GC...C....T.............
Mt.Ranginui(2)                       TT.A..A..C..A..A...A....G.GC...C....T.............
Whareorino                           TT.A..A..C..A..A...A....G.GC...C....T.............
HunuaMts.                            TT.A..A..C..A..A...A....G.GC...C....T.............
Manganuku                            TT.A..A..C..A..A...A....G.GC...C....T.............
Toatoa                               TT.A..A..C..A..A...A....G.GC...C....T.............
Whitikau(1)                          TT.A..A..C..A..A...A....G.GC...C....T.............
Whitikau(2)                          TT.A..A..C..A..A...A....G.GC...C....T.............
Whanarua_Ruatoria(1)                 TT.A..A..C..A..A...A....G.GC...C....T.............
Ruatoria(2)                          TT.A..A..C..A..A...A....G.GC...C....T.............

[                                    310        320        330        340        350]
[                                    .          .          .          .          .]
L.archeyi(1)                         GGGGATTCTCCGTAGATAACGCAACCCTAACCCGGTTCTTTGCCTTCCAT
L.archeyi(2)                         ..................................................
Waitakere(1)                         ....C.....T.....C..T.............C.....CA.T..T...
Waitakere(2)                         ....C.....T.....C..T.............C.....CA.T..T...
GreatBarrierIs(1) Waitakere(3)       ....C.....T.....C..T.............C.....CA.T..T...
GreatBarrierIs(2)                    ....C.....T.....C..T.............C.....CA.T..T...
TokateaRidge                         ....C.....T.....C..T.............C.....CA.T..T...
Mt.Moehau(1)                         ....C.....T.....C..T.............C.....CA.T..T...
Mt.Moehau(2)                         ....C.....T.....C..T.............C.....CA.T..T...
Northland                            ....C.....T.....C..T.............C.....CA.T..T...
Brynderwyn(2)                        ....C.....T.....C..T.............C.....CA.T..T...
Waipu(2)                             ....C.....T.....C..T.............C.....CA.T..T...
Tapu(1)                              .A..C.....T.....C..T.............C.....CA.T..T...
Tapu(2)                              .A..C.....T.....C..T.............C.....CA.T..T...
GoldenCross(1)                       .A..C.....T.....C..T.............A.....CA.T..T...
GoldenCross(2)                       .A..C.....T.....C..T.............A.....CA.T..T...
GoldenCross(3)                       .A..C.....T.....C..T.............A.....CA.T..T...
Mt.Ranginui(1)                       .A..C.....T.....C..T.............C.....CA.T......
Mt.Ranginui(2)                       .A..C.....T.....C..T.............C.....CA.T......
Whareorino                           .A..C.....T.....C..T.............C.....CA.T..T...
HunuaMts.                            .A..C.....T.....C..T.............C.....CA.T..T...
Manganuku                            ....C.....T.....C..T.............C.....CA.T..T...
Toatoa                               ....C.....T.....C..T.............C.....CA.T..T...
Whitikau(1)                          ....C.....T.....C..T.............C.....CA.T..T...
Whitikau(2)                          ....C.....T.....C..T.............C.....CA.T..T...
Whanarua_Ruatoria(1)                 ....C.....T.....C..T.............C.....CA.T..T...
Ruatoria(2)                          ....C.....T.....C..T.............C.....CA.T..T...
```

```
[                                    360        370        380        390        400]
[                                    .          .          .          .          .]
L.archeyi(1)                         TTCCTTCTGCCATTTATGATCGCAGGCGCCACTATTCTACACCTCATTTT
L.archeyi(2)                         ..T.....................................C....
Waitakere(1)                         ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Waitakere(2)                         ........T..T..CC.A.CA.T......A.....CA.C......T.A..
GreatBarrierIs(1) Waitakere(3)       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
GreatBarrierIs(2)                    ........T..T..CC.A.CA.T......A.....CA.C......T.A..
TokateaRidge                         ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Mt.Moehau(1)                         ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Mt.Moehau(2)                         ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Northland                            ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Brynderwyn(2)                        ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Waipu(2)                             ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Tapu(1)                              ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Tapu(2)                              ........T..T..CC.A.CA.T......A.....CA.C......T.A..
GoldenCross(1)                       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
GoldenCross(2)                       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
GoldenCross(3)                       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Mt.Ranginui(1)                       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Mt.Ranginui(2)                       ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Whareorino                           ........T..T..CC.A.CA.T......A.....CA.C......T.A..
HunuaMts.                            ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Manganuku                            ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Toatoa                               ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Whitikau(1)                          ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Whitikau(2)                          ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Whanarua Ruatoria(1)                 ........T..T..CC.A.CA.T......A.....CA.C......T.A..
Ruatoria(2)                          ........T..T..CC.A.CA.T......A.....CA.C......T.A..

[                                    410        420        430        440        450]
[                                    .          .          .          .          .]
L.archeyi(1)                         CCTACACGAGACAGGGTCAAACAACCCAACAGGATTAAACTCAAATCCTG
L.archeyi(2)                         ....................T.........................G.
Waitakere(1)                         T..G.....A..C..A.....T............C..........C....
Waitakere(2)                         TT.G.....A..C..A.....T............C..........C....
GreatBarrierIs(1) Waitakere(3)       T..G.....A..C..A.....T............C..........C....
GreatBarrierIs(2)                    T..G.....A..C........T.....G......C..........C....
TokateaRidge                         T..G.....A..C..A.....T............C..........C....
Mt.Moehau(1)                         T..G.....A..C..A.....T............C..........C....
Mt.Moehau(2)                         T..G.....A..C..A.....T............C..........C....
Northland                            T..G..T..A..C..A.....T............C..........C....
Brynderwyn(2)                        T..G..T..A..C..A.....T............C..........C....
Waipu(2)                             T..G..T..A..C..A.....T............C..........C....
Tapu(1)                              T..G.....A..C..A.....T............C..........C....
Tapu(2)                              T..G.....A..C..A.....T............C..........C....
GoldenCross(1)                       T........A..C..A.....T............C..........C....
GoldenCross(2)                       T........A..C..A.....T............C..........C....
GoldenCross(3)                       T........A..C..A.....T............C..........C....
Mt.Ranginui(1)                       T..G.....A..C..A.....T............C..........C....
Mt.Ranginui(2)                       T..G.....A..C..A.....T............C..........C....
Whareorino                           T..G.....A..C..A.....T............C..........C....
HunuaMts.                            T..G.....A..C..A.....T............C..........C....
Manganuku                            T..G.....A..C..A.....T............C..........C....
Toatoa                               T..G.....A..C..A.....T............C..........C....
Whitikau(1)                          T..G.....A..C..A.....T............C..........C....
Whitikau(2)                          T..G.....A..C..A.....T............C..........C....
Whanarua Ruatoria(1)                 T..G.....A..C..A.....T............C..........C....
Ruatoria(2)                          T..G.....A..C..A.....T............C..........C....
```

```
[                                   460        470        480        490        500]
[                                   .          .          .          .          .]
L.archeyi(1)                        ACAAAGTAACTTTCCACCCCTATTTTTCCTATAAAGACCTCCTAGGCTTC
L.archeyi(2)                        .............................................
Waitakere(1)                        .T.....CC.C........A.....C..T..C........A.........
Waitakere(2)                        .T.....CC.C........A.....C..T..C........A.........
GreatBarrierIs(1) Waitakere(3)      .T.....CC.A........A.....C..T..C......T.A.........
GreatBarrierIs(2)                   .T.....CC.C........A.....C..T..C......T.A.........
TokateaRidge                        .T.....CC.C........A.....C..T..C......T.A.........
Mt.Moehau(1)                        .T.....CC.C........A.....C..T..C......T.A.........
Mt.Moehau(2)                        .T.....CC.C........A.....C..T..C......T.A.........
Northland                           .T.....CC.C........A.....C..T..C......T.A.........
Brynderwyn(2)                       .T.....CC.C........A.....C..T..C......T.A.........
Waipu(2)                            .T.....CC.C........A.....C..T..C......T.A.........
Tapu(1)                             .T.....CC.A........A.....C..T..C......T.A.........
Tapu(2)                             .T.....CC.A........A.....C..T..C......T.A.........
GoldenCross(1)                      .T.....CC.C........AA....C..T..C......T.A.........
GoldenCross(2)                      .T.....CC.C........A.....C..T..C......T.A.........
GoldenCross(3)                      .T.....CC.C........A.....C..T..C......T.A.........
Mt.Ranginui(1)                      .T.....CC.C........A.....C..T..C......T.A.........
Mt.Ranginui(2)                      .T.....CC.C........A.....C..T..C......T.A.........
Whareorino                          .T.....CC.C........A.....C..T..C......T.A.........
HunuaMts.                           .T.....CC.C........A.....C..T..C......T.A.........
Manganuku                           .T.....CC.C........A.....C..T..C......T.A.........
Toatoa                              .T.....CC.C........A.....C..T..C......T.A.........
Whitikau(1)                         .T.....CC.C........A.....C..T..C......T.A.........
Whitikau(2)                         .T.....CC.C........A.....C..T..C......T.A.........
Whanarua Ruatoria(1)                .T.....CC.C........A.....C..T..C......T.A.........
Ruatoria(2)                         .T.....CC.C........A.....C..T..C......T.A.........
```

```
[                                   510        520        530        540        550]
[                                   .          .          .          .          .]
L.archeyi(1)                        TACATAATAATTGTTACCCTGGGCCTTCTAGCTTTATTTTCCCCAAACCT
L.archeyi(2)                        ................T.............................
Waitakere(1)                        .....T.....CACCT..A.AT..T.A.....CC.....G....G..T..
Waitakere(2)                        .....T.....CACCT..A.AT..T.A.....CC.....G....G..T..
GreatBarrierIs(1) Waitakere(3)      .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
GreatBarrierIs(2)                   .....T.....CACCT..A..T..T.A.....CC.....G.......T..
TokateaRidge                        .....T......ACCT..A.AT..T.A.....CC.....G.......T..
Mt.Moehau(1)                        .....T......ACCT..A.AT..T.A.....CC.....G.......T..
Mt.Moehau(2)                        .....T......ACCT..A.AT..T.A.N...CC.....G.......T.?
Northland                           .....T.....CACCT..A.AT....A.....CC.....G.......T..
Brynderwyn(2)                       .....T.....CACCT..A.AT....A.....CC.....G.......T..
Waipu(2)                            .....T.....CACCT..A.AT....A.....CC.....G.......T..
Tapu(1)                             .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
Tapu(2)                             .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
GoldenCross(1)                      .....T.....CACCT..A.AT..T.A.....CCN....G.......T..
GoldenCross(2)                      .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
GoldenCross(3)                      .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
Mt.Ranginui(1)                      .....T.....CACCT..A.AT....A.....CC.....G.......T..
Mt.Ranginui(2)                      .....T.....CACCT..A.AT....A.....CC.....G.......T..
Whareorino                          .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
HunuaMts.                           .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
Manganuku                           .....T.....CACCT..G.AT..T.A.....CC.....G.......T..
Toatoa                              .....T.....CACCT..G.AT..T.A.....CC.....G.......T..
Whitikau(1)                         .....T.....CACCT..G.AT..T.A.....CC.....G.......T..
Whitikau(2)                         .....T.....CACCT..G.AT..T.A.....CC.....G.......T..
Whanarua Ruatoria(1)                .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
Ruatoria(2)                         .....T.....CACCT..A.AT..T.A.....CC.....G.......T..
```

```
[                                    560        570        580        590        600]
[                                    .          .          .          .          .]
L.archeyi(1)                         CTTAGGAGACCCAGAAAATTTCACCCCTGCAAACCCATTAATTACCCCAC
L.archeyi(2)                         .....................................................
Waitakere(1)                         TC.................T........C..T...C..G....T....
Waitakere(2)                         TC.................T........C..T...C..G....T....
GreatBarrierIs(1) Waitakere(3)       TC.................T........C..T...C..G....T....
GreatBarrierIs(2)                    TC.................T........C..T...C..G....T....
TokateaRidge                         TC.................T........C..T...C..G....T....
Mt.Moehau(1)                         TC.................T........C..T...C..G....T....
Mt.Moehau(2)                         NC.................T.....N..C..T...C..G.....NNNN
Northland                            TC.................T........CG.T...C..G....T..G.
Brynderwyn(2)                        TC.................T........CG.T...C..G....T..G.
Waipu(2)                             TC.................T........CG.....C..G....T..G.
Tapu(1)                              TC.G...............T........CG.T...C..G....T..C.
Tapu(2)                              TC.G...............T........CG.T...C..G....T..C.
GoldenCross(1)                       TC.................T........CG.T...C..G.A..T....
GoldenCross(2)                       TC.................T........NG.T...N..G....T....
GoldenCross(3)                       TC.................T........CG.T...C..G....T....
Mt.Ranginui(1)                       TC.................T........CG.T...C..G.......G.
Mt.Ranginui(2)                       TC.................T........CG.T...C..G.......G.
Whareorino                           TC.................T........CG.T...C..G.......G.
HunuaMts.                            TC.................T........CG.T...C..G....T....
Manganuku                            TC.................TG.......CG.T...C..G.........
Toatoa                               TC.................TG.......CG.T...C..G.........
Whitikau(1)                          TC.................TG.......CG.T...C..G.........
Whitikau(2)                          TC.................TG.......CG.T...C..G.........
Whanarua Ruatoria(1)                 TC.................T........CG.T...C..G....T....
Ruatoria(2)                          TC.................T........CG.T...C..G....T....
                                                                  V)         V
```

Appendix D

Complete sequences for the 17 protein haplotypes. Numbers following the site names refer to the corresponding mitochondrial haplotypes. Ambiguities are marked with "?." The number of individuals represented by each haplotype is as follows:

L.archeyi(1)	1	Brynderwyn(2)	1
L.archeyi(2)	1	GoldenCross(3)	3
Common	16	HunuaMts.	5
GreatBarrierIs. Waitakere	12	Northland	6
TokateaRidge Mt.Moehau(1)	9	Waipu(2)	1
Mt.Moehau(2)	1	Mt.Ranginui(2)	1
Tapu(2)	1	Manganuku Toatoa Whitikau(1)	5
GoldenCross(1)	1	Whitikau(2)	2
GoldenCross(2)	1		

```
[                                10        20        30        40        50]
[                                .         .         .         .         .]
L.archeyi(1)                     AHICRDVNCGWLIRNMHANGASLFFICIYLHIGRGMYYGSYLFKETWNIG
L.archeyi(2)                     T.................................................
Common                           T.......Y......I..................................
GreatBarrierIs Waitakere         T.......Y......I..................................
TokateaRidge Mt.Moehau(1)        T.......Y......I..................................
Mt.Moehau(2)                     T.......Y......I..?...............................
Tapu(2)                          T.......Y......I......?...........................
GoldenCross(1)                   ........Y......I..................................
GoldenCross(2)                   .?......Y......I..................................
Brynderwyn(2)                    T.......Y......I..................................
GoldenCross(3)                   ........Y......I..................................
HunuaMts.                        ........Y......I.............................S..
Northland                        T.......Y......I..................................
Waipu(2)                         T.......Y......I..................................
Mt.Ranginui(2)                   T.......Y......I..?...............................
Manganuku Toatoa Whitikau(1)     T.......Y......I..................................
Whitikau(2)                      T.......?......I..................................
```

```
[                                60        70        80        90        100]
[                                .         .         .         .         .]
L.archeyi(1)                     VVLLFLVMATAFVGYVLPWGQMSFWGATVITNLLSAIPYVGNTMVQWIWG
L.archeyi(2)                     .................................................
Common                           .?.....................................M..I.S.L......
GreatBarrierIs Waitakere         .?.....................................M..I.S.L......
TokateaRidge Mt.Moehau(1)        .?.....................................M..I.G.L......
Mt.Moehau(2)                     .?.....................................M..I.G.L......
Tapu(2)                          .?.....................................M..I.S.L......
GoldenCross(1)                   .?.................?...................M..I.G.L......
GoldenCross(2)                   .?.................?...................M..I.G.L......
Brynderwyn(2)                    .?.....................................M..I.G.L......
GoldenCross(3)                   .?.....................................T..I.D.L......
HunuaMts.                        .?.....................................M..I.S.L......
Northland                        .?.....................................M..I.G.L......
Waipu(2)                         .?.................?...................M..I.G.L......
Mt.Ranginui(2)                   .?.....................................M..I.S.L......
Manganuku Toatoa Whitikau(1)     .?.....................................M..I.S.L......
Whitikau(2)                      .?.....................................M..I.S.L......
```

```
[                                  110        120        130        140        150]
[                                  .          .          .          .          .]
L.archeyi(1)                       GFSVDNATLTRFFAFHFLLPFMIAGATILHLLFLHETGSNNPTGLNSNPD
L.archeyi(2)                       ..............................I.................
Common                             ............T.......LTV....I.....................
GreatBarrierIs Waitakere           ............T.......LTV....I.....................
TokateaRidge Mt.Moehau(1)          ............T.......LTV....I.....................
Mt.Moehau(2)                       ............T.......LTV....I.....................
Tapu(2)                            ............T.......LTV....I.....................
GoldenCross(1)                     ............T.......LTV....I.....................
GoldenCross(2)                     ............T.......LTV....I.....................
Brynderwyn(2)                      ............T.......LTV....I.....................
GoldenCross(3)                     ............T.......LTV....I.....................
HunuaMts.                          ............T.......LTV....I.....................
Northland                          ............T.......LTV....I.....................
Waipu(2)                           ............T.......LTV....I.....................
Mt.Ranginui(2)                     ............T.......LTV....I.....................
Manganuku Toatoa Whitikau(1)       ............T.......LTV....I.....................
Whitikau(2)                        ............T.......LTV....I.....................

[                                  160        170        180        190        ]
[                                  .          .          .          .          ]
L.archeyi(1)                       KVTFHPYFSYKDLLGFYMMIVILGLLALFSPNLLGDPENFTPANPLITP
L.archeyi(2)                       ..................T.........................
Common                             ..P.............I..TSMC.....A.............D..V..
GreatBarrierIs Waitakere           ..P.............I..TSMC.....A.............V..
TokateaRidge Mt.Moehau(1)          ..P.............I..TSMC.....A.............V..
Mt.Moehau(2)                       ..P.............I..TSMC.?...A..?..........V.?
Tapu(2)                            ..P.............I..TSMC.....A.............D..V..
GoldenCross(1)                     ..P...N.........I..TSMC...?.A.............D..V..
GoldenCross(2)                     ..P.............I..TSMC.....A.............D.?V..
Brynderwyn(2)                      ..P.............I..TSMC...L.A.............D..V..
GoldenCross(3)                     ..P.............I..TSMC.....A.............D..V..
HunuaMts.                          ..P.............I..TSMC.....A.............D..V..
Northland                          ..P.............I..TSMC.....A.............D..V..
Waipu(2)                           ..P.............I..TSMC.....A.............D..V..
Mt.Ranginui(2)                     ..P.............I..TSMC.....A.............D..V..
Manganuku Toatoa Whitikau(1)       ..P.............I..TSVC.....A.........A..D..V..
Whitikau(2)                        ..P.............I..TSVC.....A.........A..D..V..
```

Appendix E

This appendix contains a matrix of the pairwise nucleotide differences between *Leiopelma hochstettri* cytochrome b haplotypes. The numbers in the matrix correspond to the following mitochondrial haplotypes:

1	TokateaRidge	15	Northland
2	Brynderwyn(2)	16	Tapu(1)
3	Whareorino	17	Toatoa
4	L.archeyi(2)	18	GoldenCross(3)
5	Waitakere(2)	19	Ruatoria(2)
6	GreatBarrierIs(1) Waitakere(3)	20	HunuaMts.
7	GoldenCross(2)	21	Tapu(2)
8	GreatBarrierIs(2)	22	Whitikau(1)
9	Mt.Ranginui(1)	23	L.archeyi(1)
10	Mt.Ranginui(2)	24	GoldenCross(1)
11	Manganuku	25	Waipu(2)
12	Mt.Moehau(1)	26	Waitakere(1)
13	Mt.Moehau(2)	27	Whitikau(2)
14	Whanarua Ruatoria(1)		

1	2	3	4	5	6	7	8	9	10	11	12	13
0												
9	0											
8	7	0										
125	126	125	0									
11	14	11	120	0								
9	16	13	125	16	0							
10	11	8	122	15	15	0						
9	16	13	126	16	8	17	0					
11	8	3	124	14	16	11	16	0				
10	7	2	123	13	15	10	15	1	0			
10	11	6	124	13	15	12	15	9	8	0		
1	10	9	126	12	10	11	10	12	11	11	0	
2	10	7	124	13	11	12	11	10	9	10	1	0
5	6	3	124	8	10	7	10	6	5	5	6	7
7	2	5	126	12	14	9	14	6	5	9	8	8
13	13	8	126	16	14	13	18	11	10	13	14	14
9	10	5	125	12	14	11	14	8	7	1	10	9
12	13	10	123	17	17	2	19	13	12	14	13	14
6	7	4	124	9	11	8	11	7	6	6	7	8
9	10	5	124	12	14	7	14	8	7	9	10	11
14	14	9	127	17	15	13	19	12	11	14	15	15
10	11	6	124	13	15	12	15	9	8	2	11	10
122	123	122	9	117	122	121	123	121	120	121	123	121
12	13	10	125	17	17	2	19	13	12	14	13	14
8	3	6	125	13	15	10	15	7	6	10	9	9
9	12	9	120	2	14	13	14	12	11	11	10	11
11	12	7	125	14	16	13	16	10	9	3	12	11

14	15	16	17	18	19	20	21	22	23	24	25	26
	27											
0												
4	0											
8	11	0										
4	8	12	0									
9	11	15	13	0								
1	5	9	5	10	0							
4	8	10	8	9	5	0						
9	12	1	13	16	10	11	0					
5	9	11	1	14	6	9	12	0				
121	123	123	122	122	121	123	124	121	0			
9	11	15	13	4	10	9	16	14	124	0		
5	1	12	9	12	6	9	12	10	122	12	0	
6	10	14	10	15	7	10	15	11	117	15	11	0
6	10	12	2	15	7	10	13	1	122	15	11	12
	0											

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